The Role of Productive Replication in the Pathogenesis of Murine Gammaherpesvirus-68

Andrew Graham Colin Barnes

(Supervisors: Professor A. A. Nash and Doctor J. Fazakerley)

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Appendix

Results

Thesis abstract

Murine gamaherpesvirus-68 (MHV-68) is natural pathogen of small free living rodents. Like Epstein-Barr virus, MHV-68 is B-cell tropic with respect to latency and sensitive to the anti-viral acyclovir (ACV). The aim of this project was to evaluate the potency of a new anti-viral called 2'-deoxy-5-ethyl-β-4'-thiouridine (4'-s-EtdU) as an inhibitor of MHV-68 replication both *in vitro* and in *vivo*. Finally to utilise the anti-viral activity of 4'-s-EtdU, to be able to study disease associated with gammaherpesvirus latency in immunocompromised mice, without the complications associated with lytic virus replication. The potency of 4'-s-EtdU as an inhibitor of the lytic replication was determined to be 15 times greater than ACV by EC_{50} assay, giving a value of 0.13μM (35ng/ml). Treatment of infected cell lines lead to the elimination of detectable productive viral replication. However, virus genomic DNA, which remained capable of reactivation after treatment withdrawal, was not eliminated. Not only was this true for cell lines which MHV-68 is known to establish latency, but also cell lines previously known only to support lytic replication. The nature of the viral persistence appeared different for the different cell lines both with respect to virus reactivation after withdrawal of treatment and to the spontaneous generation of 4'-s-EtdU resistant viral variants. The data leads to the possibility of MHV-68 being capable of episomal maintenance whilst in a perpetual state of attempted productive replication. Spontaneously arising 4'-s-EtdU resistant virus variants were cloned and isolated and found to remained sensitive to the anti-viral effects of ACV. However, further characterisation of these viral variants was not undertaken. Treatment of mice with 4'-s-EtdU (0.3mg/ml in drinking water) from 3 days post infection (in), rapidly eliminated productive virus replication in the lung tissue, but failed to prevent the establishment and long-term maintenance of viral latency in the lymphoid compartments. Treatment also failed to prevent the post acute splenomegaly commonly observed in MHV-68 infected mice. However, prophylactic treatment of mice prior to infection (in), did prevent virus dissemination to the spleen as well as preventing splenomegaly. Viral DNA remained absent from the spleen for as long as treatment was maintained, up to 54

days post infection, as determined by both co-cultivation assay and nested PCR. However, virus DNA was detected in the lung tissue of the same mice. Dissemination of virus to the spleen, on withdrawal of treatment correlated with the production of MHV-68 specific antibodies, as determined by whole virus ELISA. The experiments provided evidence for both chronic and latent infections of the lung tissue of the mice infected via the intra-nasal route. The evidence demonstrated the importance of productive virus replication in the establishment of viral latency in circulating B-cells and hence development of IM like syndromes even when MHV-68 was administered via the intra-peritoneal route. Although 4'-s-EtdU treatment protected SCID mice from an otherwise lethal MHV-68 infection, it was not possible to determine the role of productive virus replication in oncogenesis since lymphomas failed to develop in both long-term infected transiently immunosuppressed, C57BL/6 mice and in SCID mice recipients of syngeneic splenocytes. The study failed to reproduce the earlier studies (Sunil-Chandra *et al,* 1993). Surprisingly, viral latency appeared to be maintained in normal healthy BALB/c mice more efficiently than in SCID mice transiently repopulated with BALB/c splenocytes. Hence it was not possible to investigate either the therapeutic benefit or establish the role of productive virus replication in the development of MHV-68 associated tumours.

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Declaration

I hereby declare that the dissertation entitled "The Role of Productive Replication in the Pathogenesis of Murine Gammaherpesvirus-68" is not substantially the same as any I have submitted for a degree, diploma or other qualification. The dissertation is a result of my own work, and includes nothing which is the outcome of done in collaboration , except where duly acknowledged.

Signed

Date..... 01/04/99 .....

Abbreviations

3CT	2'-deoxy-3'-thiacytidine
4'-s-EtdU	2'-deoxy-5-ethyl-β-4'-thiouridine
ACV	acyclovir
AHV	Alcelaphine herpesvirus
AIDS	acquired immunodeficiency syndrome
ara A	vidarabine
AZT	zidovudine
BBS	Borate buffered saline
BCBL	body cavity-based lymphoma
B-cell	B-lymphocyte
BHK	Baby hamster kidney
BHV	Bovine herpesvirus
BL	Burkitt's lymphoma
BLN	Basal lymph node derived lymphoma
BNX	beige/nude/xid
BVaraU	1-β-D-arabinofuranosyl-(E)-5-(2-bromovinyl) uracil
BVdU	(E)-5-(2-bromovinyl)-2'-deoxy-uridine
CCV	Channel catfish virus
CD	Cluster of differentiation
cdk	cycle dependent kinase
CMV	cytomegalovirus
Cp	Bam C promoter
CR	complement receptors
CRP	complement regulatory proteins
CsA	Cyclosporin A
CTL	cytotoxic T-lymphocyte
d4T	stavadin
ddC	dideoxycytodine

DHFR	dihydrofolate reductase
ddI	didanosine
DMEM	Dulbecco's modified Eagle medium
DMSO	Dimethyl sulfoxide
dNTP	deoxyribonucleotide triphosphate
DNA	Deoxyribonucleic acid
ds	double stranded
dUTPase	deoxyuridine triphosphatease
EBER	Epstein-Barr encoded RNA
EBNA	Epstein-Barr nuclear antigen
EBNA LP	EBNA leader protein
eBL	endemic Burkitt's lymphoma
EBV	Epstein-Barr virus
EC_{50}	Effective concentration 50%
ECT10	BHK growth medium
EDTA	Ethylenediaminetetra-acetic acid (disodium salt)
EHV	equine herpesvirus
ELISA	Enzyme-Linked Immunosorbent Assay
ER	endoplasmic reticulum
FACS	flourecent antibody cell sorter
FCS	Foetal calf serum
FCV	famciclovir
FGARAT	*N*-formylglycinamide ribonucleotide amidotransferase
FIM	Fatal infectious mononucleosis
FITC	Flouroscene
Fp	Bam F promoter
g	gram
gB(C/D/H/L)	glycoprotein B (C, D, H or L)
GCR	G protein coupled receptor
GCV	gancyclovir
GMEM	Glasgow modified Eagle medium

gp	Glycoprotein
HBV	hepatitis B virus
HCl	hydrochloric acid
HCMV	Human cytomegalovirus
HHV	human herpesviruses
HIV	Human immunodeficiency virus
HOL	hairy oral leukoplakia
HPMPC	cidofovir
HRP	horseradish peroxidase
HSV	Herpes simplex virus
HSUR	HVS U-RNA
HV	herpesvirus
HVS	herpesvirus Saimiri
HVT	Turkey herpesvirus
ICAM	intercellular adhesion molecule
IDU	5'-iodo-2'-deoxyuridine
IgA(E/G/M)	immunoglobulin A (E, G or M)
IRF	interferon regulatory factor
IL	Interleukine
IM	Infectious mononucleosis
in	intra-nasal
INF	interferon
ip	intra-peritoneal
IR	internal repeat
IUdR	Iodo-2'-deoxyuridine
iv	intra-venous
kb	kilobase pair
KCl	potassium chloride
KH2PO4	mono potassium orthophosphate
KS	Kaposi's sarcoma
KSHV	Kaposi's sarcoma associated herpesvirus

l	litre
LAK	lyphokine activated killer cells
LAT	latency associated transcript
LCL	lymphoblastoid cell line
LCLC	Lymphoma cell line culture medium
LCMV	lymphocytic choriomeningitis virus
LFA	lymphocyte function-associated antigen
LMP	latent membrane protein
LPD	lymphoproliferative disease
LUR	Long unique region
μg	microgram
μMT	B-cell knock-out
μl	microlitre
M	Molar (moles per litre)
MCD	multicentric Castleman's disease
MCF	malignant catarrhal fever
MCP	major capsid protein
MDV	Marek's disease herpesvirus
MERV	MGC7 derived 4'-s-EtdU resistant virus
mg	milligram
MGC7	Mixed glial culture clone 7
MHC	major histocompatability complex
MHV-68	Murine gammaherpesvirus (strain 68)
MIP	macrophage inflammatory protein
ml	millilitre
MLN	mesenteric lymph node derived lymphoma
mm	millimetre
MOI	Multiplicity of infection
$N_2(l)$	liquid nitrogen
NaCl	sodium chloride
Na2HPO4	mono sodium orthophosphate

n-BA	n-Butyric acid
NCS	New-born calf serum
NHL	non-Hodgkin's lymphoma
NK	natural killer
NPC	nasopharyngeal carcinoma
OD_{490}	optical density at 490nm wavelength
OHV	Ovine herpesvirus
OPD	O-phenylenediamine dihydrochloride
ORF	Original reading frame
ori P	origin of plasmid replication
PAA	phosphonoacetic acid
PAN-RNA	polyadenylated nuclear localising RNA
PBS	Phosphate buffered saline
PBL	peripheral blood leukocytes
PBMC	peripheral blood mononuclear cell
PCR	Polymerase chain reaction
PCV	penciclovir
PEL	primary effusion lymphoma
PFA	foscarnet
pfu	plaque forming units
Rb	Retinoblastoma tumour suppresser
RPM	revolutions per minute
RNA	ribonucleic acid
RR	ribonucleotide reductase
RT	room temperature
sBL	sporadic Burkitt's lymphoma
SCID	Severely Combined Immunodeficient
SDW	sterile distilled water
SP-	Spleen derived lymphoma
ssDBP	single stranded DNA binding protein
SUR	short unique region

Stp	saimiri transformation-associated protein
TAE	tris-acetate ETDA
T-cell	T-lymphocyte
TFT	triflurothymidine
TGF	tumour growth factor
Tip	tyrosine kinase interacting protein
TK	thymidine kinase
TNE	tris normal saline ETDA
TPA	12-O- tetradecanoylphorbol 13-acetate
TPB	Tryptose phosphate broth
TR	Terminal direct repeat
TS	thymidine synthase
UCR	unique coding region
UV	ultra violet
Wp	BamW promoter
VACV	valiciclovir
V/V	volume per volume
VZV	Varicella-zoster virus
W/V	weight per volume
XLP	X-linked lymphoproliferative syndrome
ZEBRA	Z fragment Epstein-Barr reactivation antigen

Table appendix

1.1.1 The herpesviruses

Herpesviruses are a ubiquitous group of highly infectious large enveloped viruses with a genome comprised of deoxyribonucleic acid (DNA). Herpesviruses have been isolated from all major vertebrate groups, such as fish, amphibians, reptiles, birds and mammals (*see Table 1.1*). To date there have been 8 herpesviruses isolated, the natural host of which is man. The human herpesviruses (HHV) have been designated HHV1 to 8 but are commonly referred to as herpes simplex virus (HSV) 1 & 2, Varicella-zoster virus (VZV), human cytomegalovirus (CMV or HCMV), Epstein-Barr virus (EBV), HHV6, HHV7 and Kaposi's sarcoma associated herpesvirus (KSHV), respectively. Primary infection can lead to the development of diseases such as chicken pox, with VZV, and infectious mononucleosis (IM), with EBV, however most primary infections are asymptomatic. Viral infection of the host is life long, a feature common to all herpesviruses, and can lead to spontaneous recurrent disease, such as cold sores with HSV and shingles with VZV. Virus levels are controlled by the host's immune system and immunosuppression can lead to fatal diseases such as disseminating infection with HCMV, lymphoproliferative (LPD) disease, with EBV and ocular disease with HSV. Herpesviruses have also been associated with a number of malignancies such as endemic Burkitt's lymphoma (eBL) and nasopharyngeal carcinoma (NPC), with EBV and Kaposi's sarcoma (KS), with KSHV.

1.1.2 Herpesvirus structure and genome architecture

Herpesvirus particles consist of a linear double stranded (ds) DNA genome contained within an icosahedral capsid comprised of 162 capsomeres. The capsid is enclosed within a glycoprotein embedded lipid envelope, separated by an amorphous protein tegument (Roizman *et al*, 1996). The viral genomes range, in size from 125 kilobase pairs (kb) for VZV to 230kb for HCMV, and in G+C content (molar ratio) from 42%

Table 1.1* **Examples of vertebrate herpesviruses that have been isolated and at least partially characterised.**

Designation	Common name	Genome	
		G+C (%)	Size (kb)
Piscean herpesviruses			
Ictalurid herpesvirus 1	Channel catfish HV	56	130
Salmonid herpesvirus 1	HV salmonis		
Cypinid herpesvirus	Carp pox HV		
Amphibian herpesviruses			
Ranid herpesvirus 1	Lucke frog HV	46	
Ranid herpesvirus 2	Frog HV 4	56	
Reptilian herpesviruses			
Iguanid herpesvirus 1	Green iguana HV		
Elapid herpesvirus	Indian cobra HV		
Avian herpesviruses			
Gallid herpesvirus 1	Marek's disease HV	47	180
Phalacrocoracid herpesvirus1	Cormorant HV	58	
Strigid herpesvirus 1	Owl hepatosplenitis virus	61	
Mammalian herpesviruses			
Macropodid herpesvirus 2	Dorcopsis wallaby HV	50	135
Murid herpesvirus 1	Mouse cytomegalovirus	59	235
Leporid herpesvirus 1	HV sylvilagus	33	145
Suid herpesvirus 1	Pseudorabies virus	74	140
Equid herpesvirus 1	Equine abortion HV	57	142
Bovine herpesvirus 1	Infectious bovine rhinotracheitis HV	72	140
Felid herpesvirus 1	Feline rhinotracheitis HV	46	135
Aotine herpesvirus 1	HV aotus 1	55	220

* *Table adapted from* Roizman *et al*, 1996.

in HHV6 to 75% in pseudorabies virus (Roizman *et al*, 1996). There are a number of different genomic structural arrangements commonly found within the *Herpesviridae*. These have been termed type 'A' to 'F' and denote, in order of complexity, the number, positions and relative orientations of repeat sequences as well as unique or quasi unique coding regions, within the viral genome. Type A arrangements are found in herpesviruses such as Channel catfish virus (CCV), believed to represent an ancestral herpesvirus group, the gammaherpesvirus equine herpesvirus (EHV) 2 and the betaherpesvirus subgroup *Roseoloviruses*, which includes HHV 6 & 7. B type arrangements are found in many γ-2 herpesviruses (*Rhadinoviruses*), such as herpesvirus (HV) Saimiri (HVS) and HV ateles. Type C arrangements are found in the γ–1 herpesviruses (*Lymphocryptoviruses*), such as EBV, HV pan and HV papio. Type D arrangements are common to the alphaherpesvirus sub-group *Varicelloviruses*, such as VZV and EHV1. Type E arrangements are common to the alphaherpesvirus subgroup *Simplexviruses*, such as HSV1 & 2 and bovine herpesvirus (BHV) 2 as well as the β–1 herpesviruses (*Cytomegaloviruses*), such as HCMV and HV aotus type 1. Type F arrangements are found in the betaherpesvirus subgroup *Muromegalovirus*, such as murine CMV 1 & 2 and tupaia herpesvirus, and the gammaherpesvirus EHV-5 (Roizman *et al*, 1996 & Telford *et al*,1993).

1.1.3 **The herpesvirus sub-families**

There are essentially 3 herpesvirus sub-families: alpha, beta and gamma. The groupings were originally organised on the basis of their biological properties, both *in vivo* and *in vitro*. This form of classification still holds true in many cases but does not allow for tropism diversity with in sub-families. Modern classification is now based on genetic determinants, such as the relative order of conserved blocks of genes, common to all herpesviruses (Chee *et al*, 1990 & Gomples *et al*, 1995).

The alphaherpesviruses generally have a variable host range, short reproductive cycle which leads to lysis of the host cells. They are generally neurotropic with respect to

in vivo latent infections (primarily in sensory ganglia) and are readily propagated in permissive cell lines, *in vitro*. Examples of species members of the *Alphaherpesvirinae* are HSV 1, HSV 2, VZV, BHV 2, EHV 1 and Marek's disease herpesvirus (MDV) 1. Human alphaherpesviruses are responsible for cold sores, chicken pox and shingles, ocular disease and encephalitis in man. MDV induces T-cell lymphomas in chickens.

The betaherpesviruses generally have a restricted host range and a long replicative cycle in tissue culture. A productive infection of cells induces cytomegalia (enlargement) and persistently infected cell lines are readily established *in vitro*. The common sites of persistence *in vivo* are secretary glands, lymphoreticular cells, the kidneys and the lungs. Members of the *Betaherpesvirinae* are HCMV, HHV 6, HHV 7 and murine CMV. Betaherpesviruses are responsible for foetal deformities, childhood roseola and disseminating infection related disease and encephalitis in immunosuppressed / immunocompromised individuals.

The gammaherpesviruses generally have a highly restricted host range, limited to the family/ order, to which the natural host belongs. Though they often replicate poorly in tissue culture, some will productively infect epithelial and fibroblastoid cells *in vitro*. Latent infections of lymphocytes can be established both *in vivo* and *in vitro* and lymphoid tissue is often the site of viral latency *in vivo*. A common feature of the subfamily is the ability to immortalise / transform lymphocytes *in vitro* and *in vivo* and have been associated with numerous lymphoproliferative and malignant conditions both in humans and animals. Examples of the *Gammaherpesvirinae* are EBV, HV papio, KSHV, HVS, HV ateles, BHV 4, Alcelaphine herpesvirus (AHV), EHV 2 & 5 and murine gammaherpesvirus 68 (MHV-68) (*see Table 1.4*).

1.1.4 **Herpesvirus conserved gene blocks**

It has been observed that homologies to a number of 'core' genes exist in all sequenced mammalian herpesviruses. These genes generally code for the

fundamental structural proteins and enzymes required for productive viral replication. The conserved genes occur in blocks and within the blocks they are generally co-linear with respect to order and orientation. Initially 3 blocks were described (Davidson *et al*, 1987) but as more sequencing data became available, these were broken up into 7 blocks (Chee *et al*, 1990). The relative order and orientation of these conserved gene blocks is specific to the 3 herpesvirus sub-groups and forms the basis of the modern classification system by which mammalian and avian herpesviruses are assigned a sub-grouping. The order and alignment of the blocks is taken from the betaherpesviruses since the β–2 herpesviruses have an 'A' type genome structure, which is believed to represent the ancestral herpesvirus genome structure. The order therefore, in which betaherpesviruses encode the conserved blocks is I, II, III, IV, V, VI, VII. For alphaherpesviruses it is III, I, II', IV',V',VI, VII and for gammaherpesviruses it is II', IV', V', VI, VII, III', I (where blocks' are encoded in the inverse direction) (Gompels *et al*, 1995).

1.1.5 **Conserved and partially conserved herpesvirus genes**

Block I contains the genes for the large subunit of the enzyme ribonucleotide reductase (RR) as well as a number of virion structural protein genes. Block II contains the genes for the herpesvirus specific DNA polymerase, glycoprotein B (gB) and the single stranded DNA binding protein (ssDBP). Blocks III, IV and V contain the genes for dUTPase, the major capsid protein (MCP) and glycoprotein H (gH), respectively. Block VI contains the genes for the viral DNA packing proteins, alkaline exonuclease and the herpesvirus specific kinase, responsible for the phosphorylation of gancyclovir (GCV). Block VII contains the genes for Uracil DNA glucosidase and glycoprotein L (gL) (*for the breakdown of the conserved viral gene blocks, see Table 1.2*).

There are also a number of partially conserved genes found in at least two of the herpesvirus sub-groups (*see Table 1.3*), such as the thymidine kinase (TK) homologue which is encoded by both alpha and gammaherpesviruses, but not the

Table 1.2* **The herpesvirus core gene homologues, in block order, as encoded by two representative α, β and γ herpesviruses.**

Gene Block	β-Herpesviruses		γ-Herpesviruses		α-Herpesviruses		Gene Function
	HCMV	**HHV6**	**HVS**	**EBV**	**VZV**	**HSV-1**	
1	UL44	UL27	ORF59	BMRF1	ORF16	UL42	DNA replication protein[2]
1	UL45	UL28	ORF61	BORF2	ORF19	UL39	RR (large)[1&2]
1	UL46	UL29	ORF62	BORF1	ORF20	UL38	Capsid assembly / DNA maturation protein[1&2]
1	UL47	UL30	ORF63	BORF1	ORF21	UL37	Myosin[1] / Tp[2]
1	UL48	UL31	ORF64	BPLF1	ORF22	UL37	Tp[1&2]
1	UL49	UL33	ORF66	BFRF2	ORF23	UL35	Cp[1]
1	UL50	UL34	ORF67	BFRF1	ORF24	UL34	Tp[2]
1	UL52	UL36	ORF68	BFLF1	ORF26	UL32	Gp[2]
1	UL53	UL37	ORF69	BFLF2	ORF27	UL31	
2	UL54	UL38	ORF9	BALF5	ORF28	UL30	DNA polymerase[1&2]
2	UL55	UL39	ORF8	BALF4	ORF31	UL27	Gp B[1&2]
2	UL56	UL40	ORF7	BALF3	ORF30	UL28	transport protein[1&2]
2	UL57	UL41	ORF6	BALF2	ORF29	UL29	ssDNA binding protein[1&2]
3	UL69	UL42	ORF57	BMLF1	ORF4	UL54	IE[2] transactivator[1]
3	UL70	UL43	ORF56	BSLF1	ORF6	UL52	Helicase/Primase[1]
3	UL71	UL44	ORF55	BSRF1	ORF7	UL51	
3	UL72	UL45	ORF54	BLLF2	ORF8	UL50	dUTPase[1&2]
3	UL73	UL46	ORF53	BLRF1	ORF9A	UL49A	Membrane/secreated protein[1]
4	UL75	UL48	ORF22	BXLF2	ORF37	UL22	Gp H[1&2]
4	UL76	UL49	ORF20	BXRF1	ORF35	UL24	fusion protein[1]
4	UL77	UL50	ORF19	BVRF1	ORF34	UL25	Tp[2]
4	UL80	UL53	ORF17	BVRF2	ORF33	UL26	Cp[2]/assembly protein[1]
5	UL85	UL56	ORF26	BDLF1	ORF41	UL18	Cp[1&2]
5	UL86	UL57	ORF25	BcLF1	ORF40	UL19	Major Cp[1&2]

Table 1.2 (continued)

Gene	β-Herpesviruses		γ-Herpesviruses		α-Herpesviruses		
Block	**HCMV**	**HHV6**	**HVS**	**EBV**	**VZV**	**HSV-1**	**Gene Function**
6	UL89 EX2	UL60	ORF29b	BDRF1	ORF42	UL15 EX2	DNA packing protein[1&2]
6	UL93	UL64	ORF32	BDLF1	ORF43	UL17	
6	UL94	UL65	ORF33	BGLF2	ORF44	UL16	
6	UL89 EX1	UL66	ORF29a	BGRF1	ORF45	UL15 EX1	DNA packing protein[1&2]
6	UL95	UL67	ORF34	BGLF3	ORF46	UL14	
6	UL97	UL69	ORF36	BGLF4	ORF47	UL13	GCV kinase[1&2]
6	UL98	UL70	ORF37	BGLF5	ORF48	UL12	Alkaline exonuclease[1&2]
6	UL100	UL72	ORF39	BGRF3	ORF50	UL10	Gp M[1&2]
6	UL102	UL74	ORF41	BBLF3	ORF52	UL8	Helicase/Primase[1&2]
6	UL103	UL75	ORF42	BBRF2	ORF53	UL7	
6	UL104	UL76	ORF43	BBRF1	ORF54	UL6	Cp[2]
6	UL105	UL77	ORF44	BBLF4	ORF55	UL5	Helicase/Primase[1&2]
7	UL114	UL81	ORF46	BKRF3	ORF59	UL2	Uracil DNA glucosidase[1&2]
7	UL115	UL82	ORF47	BKRF2	ORF60	UL1	Gp L[1&2]

** Table adapted from Gompels, et al, 1995.*

Key : ***Cp****, Capsid protein;* ***Gp****, glycoprotein;* ***Tp****, Tegument protein;* ***RR****, Ribonucleotide reductase;* ***IE****, Immediate early;* ***GCV****, Gancyclovir;* [1] *Gompels, U.A., et al, (1995) Virology 209, 29-51;* [2] *Russo, J.J., et al, (1996) Proc. Natl. Acad. Sci. USA 93, 14862-14867).*

Table 1.3* **The partially conserved herpesvirus gene homologues as encoded by two representative α, β and γ herpesviruses.**

Block	β-Herpesviruses		γ-Herpesviruses		α-Herpesviruses		Gene Function
	HCMV	HHV6	HVS	EBV	VZV	HSV-1	
1	UL51	UL35			ORF25	UL33	
1			ORF60	BaRF1	ORF18	UL40	RR (small) [2]
4			ORF21	BXLF1	ORF36	UL23	Thymidine Kinase [2]
4	UL79	UL52	ORF18	BVRF1.5			
5/6	UL87	UL58	ORF24	BcRF1			
6	UL92	UL63	ORF31	BDLF4			
6	UL96	UL68	ORF35	BGLF3.5			

* *Table adapted from Gompels, et al, 1995.*

Key: ***RR****, Ribonucleotide reductase;* [2]*Russo, J.J., et al, (1996) Proc. Natl. Acad. Sci. USA 93, 14862-14867).*

betaherpesviruses. However, most of the partially conserved genes do not code for a protein with an assigned function.

1.1.6 **Herpesvirus replication**

The herpesvirus infectious cycle, can be regarded as a multi-step process - 1/ attachment, 2/ penetration, 3/ cytoplasmic migration to the nucleus, 4/ uncoating and genomic circularisation and 5/ the establishment of latency or initiation of productive replication. Productive replication or post-latency reactivation leads to 6/ genomic replication, 7/ lytic cycle viral transcription, 8/ progeny DNA concatamer cleavage and encapsidation, 9/ glycosylation of envelope proteins, 10/ nuclear budding, 11/ cytoplasmic migration to plasma membrane and 12/ egress.

1.1.7 **Herpesvirus entry and uncoating**

Attachment occurs via viral envelope glycoprotein binding host cell receptors. The receptor for HSV is heparin sulphate (WuDunn *et al*, 1989) and the receptor for EBV is the B-lymphocyte marker CD21 (Nemerow *et al*, 1987). The viral ligand responsible for initial receptor binding of HSV to the target cell is gB and glycoprotein C (gC) (Cai *et al*, 1988). Initial binding of EBV is carried out by glycoprotein (gp) 350/220 (Nemerow *et al*, 1987). Entry into the cell can occur by direct fusion with the plasma membrane, as is the case with HSV(Morgan *et al*, 1968), where gB, glycoprotein D (gD) and gH all playing an essential role (Cai *et al*, 1988, Campadelli-finme *et al*, 1988, Desia *et al*, 1988). Conversely, entry can be mediated by endocytosis, as is the case for EBV (Tanner *et al*, 1987). Infection of peripheral B-lymphocytes is initiated by multiple gp350/220 molecules crosslink cell surface CD21. This induces a capping response from the B-cell, internalising the bound virus particle via a cellular vesicle (Tanner *et al*, 1987). Fusion between the vesicle membrane and the viral envelope is then mediated by gp85 (Miller *et al*, 1988) and in part by gp350/220. EBV can also enter cells by direct fusion, for example when super-infecting Burkitt's lymphoma (BL) derived cell lines, *in vitro*,

such as RAJI cells. However, direct fusion may not reflect any *in vivo* situations for EBV.

On entry into the cytoplasm, the herpes virion migrates through the cytoplasm to the nuclear pores (Tognon *et al*, 1981) and are probably transported by the host cell cytoskeleton (Kristensson 1986). The viral DNA is then released into the nucleoplasm where it circularises (Jacob *et al*, 1977 and 1979). In the case of EBV, the latent gene product termed EBV nuclear antigen (EBNA) 1 is necessary for episomal establishment (Reisman *et al*, 1984 and Yates *et al*, 1985).

1.1.8 **Herpesvirus productive viral genome replication**

Herpesvirus productive genome replication requires several viral encoded products for nucleotide metabolism and DNA replication. The viral genes that encode these enzymes, such as their own DNA polymerase (Davidson *et al*, 1987 and Kouzarides *et al*, 1987), and homologues to alkaline exonuclease, dUTPase and RR, comprise many of the core or partially conserved herpesvirus genes (Davidson *et al*, 1987). Genome replication is achieved by rolling circle technique producing concatamer daughter strands (Jacob *et al*, 1977 and 1979, Seigel *et al*, 1981). Capsids assemble in the nucleus and the concatameric viral DNA is cleaved and packaged to form new virions. Cleavage and packaging is coded for by consensus sequences upstream of the cleavage site. Homologous sequences exist in HSV 1 and 2, EBV, HVS, BHV1, HCMV and VZV (Davidson *et al*, 1984, Desin *et al*, 1986, Matsuo *et al*, 1984 and Spaete *et al*, 1985). The HSV and CMV cleavage and packaging sequences have been shown to be interchangeable (Spaete *et al*, 1985). The model for concatamer cleavage and packaging, called the double stranded break-gap repair model (Deiss *et al*, 1986), strictly speaking, only applies to HSV-1. Since there is such a high degree of consensus sequence homology across the *Herpesviridae*, PAC1 and PAC2 being homologous to Ub and Uc respectively, it is assumed, the other herpesviruses follow a similar pattern. The packaging complex traverses along the concatameric viral DNA, starting from any point, until it reaches the first Uc signal in Junction 1.

Concatameric scanning and packaging, into a ready assembled capsid, initiates consecutively in a Long to Short unique sequence direction. Packaging is then terminated at Junction 2, of the same concatamer. The two Junctions become aligned and juxtaposed, so the Ub and Uc sequences, of both junctions, directly face one another. The strands are cleaved, invade the homologous region of the juxtaposed strand and use it as a template to amplify the *a* region. The resultant Holliday structures are resolved and cleavage between the two *a* sequences of the two adjoining concatamers ensures neither concatameric daughter strand has an *a* sequence at both terminals (*see figure 1.1 for schematic diagram of the double stranded break gap repair model*). Encapsidation can then be completed, causing a conformational change. This allows maturation of the virion and budding through the nuclear inner lamellae (Vlazny *et al*, 1982).

1.1.9 **Herpesvirus assembly and Egress**

Glycoprotein synthesis and assimilation occurs in the cytoplasm. Glycoproteins get O- and N- linked glycosylations, though generally N- link predominate (Kari *et al*, 1988, Serafini-cessi *et al*, 1988 & 1989) The glycoproteins embed in membranes which the virions bud into so acquiring there envelope (Gong *et al*, 1990 and Kousoulas *et al*, 1983) and completed glycoprotein processing is required for viral egress (Kousoulas *et al*, 1983). Initially, the virions enter the cytoplasm by budding into the nuclear envelope. In the case of HSV, immature glycoproteins embed into the nuclear membrane, and the mature capsids bud through the membrane acquiring both envelope and glycoproteins. The immature virus particles, then pass through the Golgi apparatus, on route to the plasma membrane, where viral glycoproteins become processed to maturity (Desai *et al*, 1988, Kousoulas *et al*, 1983). The mature viruses travel to the plasma membrane, via a Golgi derived vesicle and are released into the extra-cellular space by a process of reverse phagocytosis (Johnson *et al*, 1982).

Other herpesviruses egress via different routes. It has been hypothesised that many

Figure 1.1

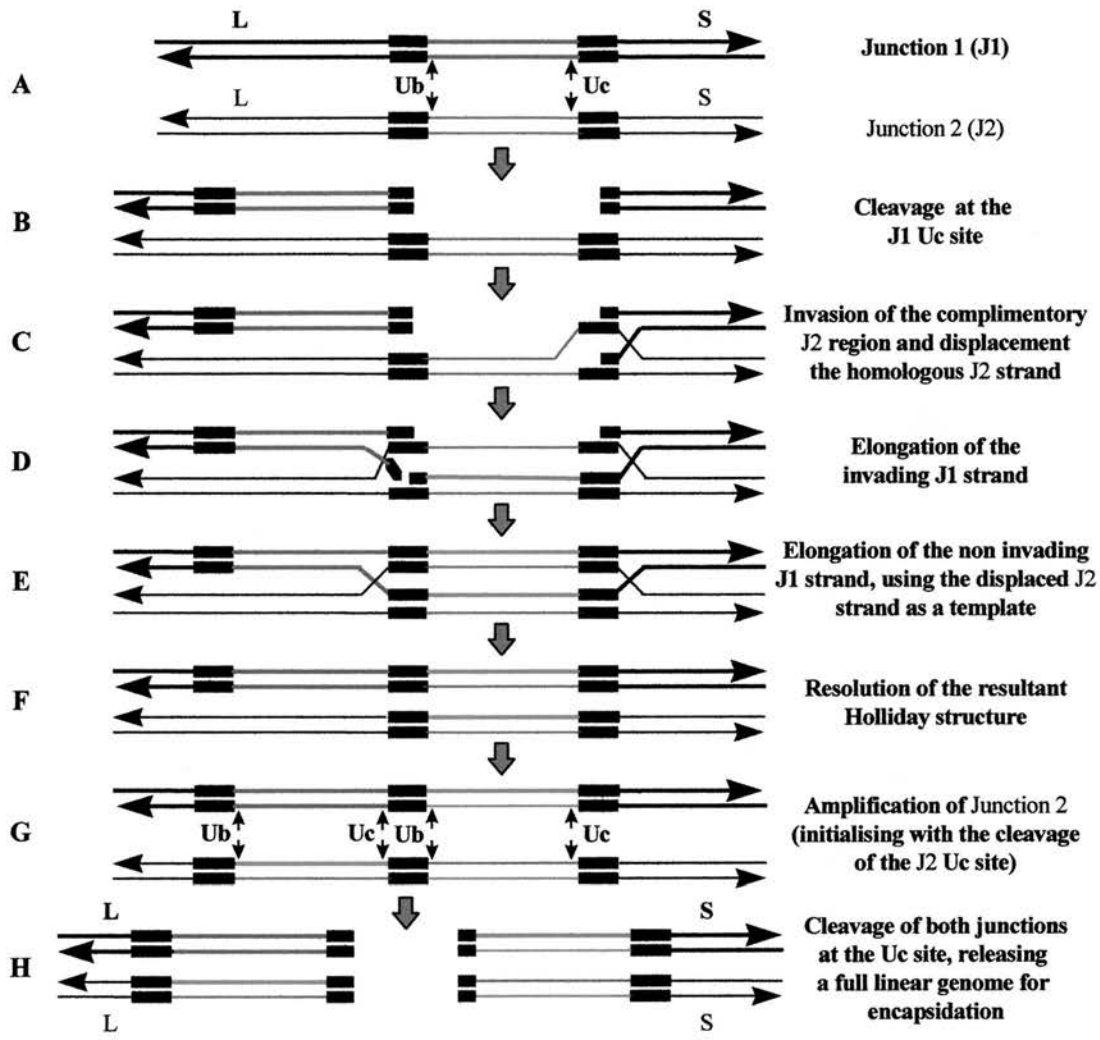


Figure 1.1 shows the molecular mechanism by which HSV converts concatameric genome daughter strands into full length linear viral genomes for packaging into virions, as described in the double stranded break-gap repair model (Deiss et al,1986). The black lines represent concatameric viral genomic DNA comprising the long unique region (L) and short unique region (S) up and down stream of the L/S junction (J) 1 & 2. Thick lines represent DNA comprising / derived from J1 and thin lines represent DNA comprising / derived from the J2. Blue lines represent the amplification region between the Ub and Uc sites and the red lines, the newly synthesis DNA. The black blocks represent the cleavage site containing sequences of J1 and 2 directly flanking the amplification region. The junction separating the long and short coding regions of the ultimate (J1) and penultimate (J2) concatameric genome segments become aligned and juxtaposed (A). A Uc directed double stranded cleavage occurs within the DR1 element of J1 (B). The S fragment 3' end then invades the homologous region of J2, displacing the J2 5' to 3' strand and annealing to the complementary J2 3' to 5' strand (C). Using the J2 3' to 5' strand as a template, the invading J1 strand elongates until it reaches the J2 Ub directed site in DR2 (D). The 3' end of the J1 L fragment then elongates, uses the displaced J2 strand as a template, until it reached the J2 Uc encoded stop site in DR1. The J1 3' ends are re-ligated to form a Holliday structure (E). The Holliday structure is resolved by recombination (F) the entire process is repeated, starting with the Uc directed double stranded cleavage within the DR1 element of J2 (G). Both strands are cleaved in the DR element separating the two amplification regions on each strand, (H) releasing two full length linear viral genomes two be packaged into virions.

members of the herpesvirus family become enveloped by the inner lamellae and then de-enveloped by the outer lamellae so releasing non-enveloped mature capsids into the cytoplasm (Stackpole *et al*, 1969). They become re-enveloped and egress by budding at the plasma membrane, as is the case for EBV (Gong *et al*, 1990), or into cytoplasmic vacuoles, as has been observed in many herpes species, such as HHV6, MCMV and HVS (Biberfield *et al*, 1987, Papadimitrion *et al*, 1984, Tralka *et al*, 1977). Enveloped particles are often observed in close association with membrane like structures (Schwartz *et al*, 1969) and it has been hypothesised that vesicles transport virus to the plasma membrane and then vesicle allows egress (Johnson *et al*, 1982, Desai *et al*, 1988, Papadimitrion *et al*, 1984).

1.2.1 **Herpesvirus Transmission**

Herpesvirus transmission is generally associated with chronic viral replication, often at sites of the initial infection. Although EBV predominately establishes a latent infection in B-lymphocytes, it can also establish a low level chronic infection of the oropharyngeal epithelium (Yao *et al*, 1985 & Tomas *et al*, 1991). This is particularly apparent in chronic carriers and AIDS patients. Chronic replication in the epidermal skin tissue has also been observed in HSV. Prolonged periods of virus shedding can occur asymptomatically in normal healthy sera positives or symptomatically with cold sore development.

1.2.2 **Herpesvirus persistence *in vivo*.**

One of the hall marks of herpesvirus infection *in vivo*, is their ability to establish a life long infection of the host. Persistent viral infection strategies can generally be categorised into 2 different types. The first is defined by viral persistence in association with the continual presence of productively replicating virus. This is termed a chronic productive infection and is commonly associated with the lymphocytic choriomeningitis virus (LCMV) infection of mice, and the vertically transmitted hepatitis B virus (HBV) infection of man (Buchmeier *et al*, 1980 &

Beasley *et al*, 1977). The other strategy associated with persistent viral infections is defined by the presence of viral genomes, which is capable of reactivating, but exists in the absence of infectious virus, or virion particles. This is termed as a latent infection, and is commonly associated with, and typified by, herpesvirus persistent infections *in vivo* (Ahmed *et al*, 1990). Latent herpesviruses have genomes which, unlike the linear genomes found in virions, are in a covalently closed circular form, referred to as episomes (Kaschaka-Dierich *et al* 1982, Gardella *et al*, 1984, Harris *et al*, 1988, Mellerick *et al*, 1987, Adams *et al*, 1987, Alfieri *et al*, 1991, Decker *et al* 1996 & Renne, *et al*, 1996). Gammaherpesvirus episomes replicate as a plasmid, in latently infected, dividing cells, using the cellular replication enzymes. To achieve this, viral genomes contain a plasmid origin of replication (ori P) (Yates *et al*, 1985 and Kung *et al*, 1996). This allows the virus genome to exist at a constant copy number in the cell, without the need to express productive replication associated gene products, such as the virus encoded DNA polymerase.

1.2.3 **Evasion of the host's immune system.**

To establish a life long persistent infection *in vivo*, the virus must be able to successfully evade the host's immune responses. This is largely achieved, in latent viral infections, by the virus establishing a pattern of highly restricted gene expression, in the infected cell. Latency associated, restricted gene expression has been observed in many different herpesviruses both *in vivo* and *in vitro* (Rowe *et al*, 1986, Kerr *et al*, 1992, Murthy *et al*, 1986, Stevens *et al*, 1987 & Zhong *et al* 1996). Reduced viral gene expression, greatly reduces the amount of antigen presentation at the surface of the cell, and so the infected cell goes unnoticed.

A second method of avoiding detection is to either specifically down regulate cellular receptors/ligands essential for targeting of the infected cell by the cellular immune system, or to infect cells where there expression is restricted. CD8 cytotoxic T-lymphocytes (CTL) are largely responsible for the destruction of virus harbouring cells. Infected cells are recognised, by the CTLs, by the presentation of

the acute infection travel to the sensory ganglion neuronal somas, via retrograde axonal transport, and establish latency (Cook *et al*, 1973). Latency is largely, though not exclusively, established in sensory ganglion neurones (Lonsdale *et al*, 1979). HSV1 normally establishes latency in the trigeminal ganglia, due to the oro-respiratory transmission of infection, to the oropharyngeal mucosa. HSV 2 normally establishes latency in the sacral ganglia, due to sexual transmission, of infection, to the vaginal tract or penile skin.

1.2.5 **HVS latent gene expression**

Latent HSV genomes exist in a non-integrated, episomal form, in infected neuronal nuclei (Mellerick *et al*, 1987). During latency the majority of the viral genome is transcriptionally inactive. However, a site on the opposite strand, overlapping the early (α) gene ICP0 is transcriptionally active (Stevens *et al*, 1987 & Gordon *et al*, 1988). This has been termed the latency associated transcript (LAT) region. There are 2 transcripts, transcribed from this region, major and minor LATs, and possibly a third, believed to initiate from a site further up stream. The 2 predominant transcripts, are 2.3 and 1.7 kb, respectively, the latter being derived from the former, via a splicing event (Wagner *et al*, 1988). The LATs transcripts are not believed to be polyadenylated or translated *in vivo* and are not necessary for both the establishment and maintenance of latency, *in vivo*, (Javier *et al*, 1988) but may play a role in reactivation. Reactivation of HSV is not fully understood, but is often associated with localised stimuli, such as injury to tissue innovated by neurones carrying latent virus, or more systemic stimuli, such as physical or emotional stresses. Reactivated virus travels, via peripheral nerves, to the epidermis, where a recurrent or recrudescent infection occurs. Recurrent infections will occur despite the presence of cellular and humoral immunity. A recurrent infection may lead to vesicular skin lesions and mucosal ulcerations. These lesions are generally caused by viral mediated cell death and the associated inflammatory responses (Whitley *et al*, 1990).

1.2.6 **Latency in non-neurotropic alphaherpesviruses**

MDV and Turkey herpesvirus (HVT) latently infect lymphocytes and show strain dependant transforming abilities (Calnek *et al*, 1979). Highly transforming strains of MDV are the causative agent of Marek's disease, a T-cell lymphoma of chickens. Upon infection, MDV lytically infects B-lymphocytes, often inducing a splenomegaly, and transforms, via latent infection, activated CD4 positive / CD8 negative T lymphocytes. These go on to form lymphomas. MDV positive CD4 negative / CD8 positive and CD4 negative / CD8 negative T lymphocytes will also spontaneously transform *in vitro*, when taken from chickens, with inflammatory stimuli, during early infection time points (Schat *et al*, 1991). Genetic cluster analysis of MDV and HVT has shown them to belong to the alphaherpesviruses, not the gammaherpesviruses, though they are lymphotropic in nature (Buckmaster *et al*, 1988).

1.2.7 **Betaherpesvirus persistence and latency**

Of the 3 herpesvirus sub-families, betaherpesvirus latency is least well understood. Primary infection is largely asymptomatic, except during foetal development, where it can result in birth defects. Primary infection results in the establishment of a life long persistent infection. CMV persistence is associated with a number of tissues and organs, such as bone marrow and monocytes, lung, spleen, salivary glands, heart, kidney and adrenal glands. The establishment of latency, *in vivo*, appears to be linked to the severity of the primary infection and sites of primary acute infection (Balthesen *et al*, 1993, Matthias *et al*, 1994 and Ho *et al*, 1991). Recurrent infections can occur during periods of immuno-suppression. Recurrent infections under these conditions are often severe and can result in fatal CMV disease. CMV disease is most commonly associated with bone marrow transplantation recipients, but is also seen with solid organ transplants, such as lung, liver, kidney and heart (for review see Ho *et al*, 1991). In these cases CMV recurrence is often associated with the donor organ, due to, pre-established CMV latency. CMV recurrent infection and

disease is also associated with human immunodeficiency (HIV) infected patients during the latter stages of acquired immunodeficiency syndrome (AIDS) (Hirsch *et al*, 1991). Although the specific nature and cellular targets for CMV persistence remain controversial, latent murine CMV genome has been observed in splenic stromal cells (Pomeroy *et al*, 1991).

Persistence, in part, may be down to low level chronic infections, since the presentation of the major immediate early protein, by MHC class I, can be selectively down regulated, in persistently infected cells, *in vitro* (Gilbert *et al*, 1993). This in turn greatly reduces recognition by CD8 positive T lymphocytes, which are the principal effectors, as opposed to antibody responses, in combating both primary and recurrent infections (Reddehase *et al*, 1988, Mattius *et al*, 1994 & Riddell *et al*, 1992). This mechanism of chronic persistence and immune evasion, may well represent viral persistence in monocytes and lymphocytes *in vivo*, since CMV infection results in the expression of the major immediate protein only (Rice *et al*, 1984).

1.2.8 **Gammaherpesvirus persistence and latency**

The gammaherpesviruses establish latent infections in lymphoid cells (*see Table 1.4*). The 'HVS-like' γ–2 herpesviruses of the new world monkeys, establish latency in T-cells, as do the 'AHV 1-like', malignant catarrhal fever (MCF) associated γ–2 herpesviruses. The 'EBV-like' γ–1 herpesviruses of old world primates are B-cell tropic (Kieff *et al*, 1995, Bocker *et al*, 1980, Rabin *et al*, 1978, Neubauer *et al*, 1979, Ohno *et al*, 1979) as are the γ–2 herpesviruses KSHV, EHV-2, EHV-5 and MHV-68 (Cesarman *et al*, 1995a, Mesri *et al*, 1996, Sunil-Chandra *et al*, 1992b). HV *sylvilagus* will establish latency in both T and B lymphocytes (Kramp *et al*, 1985). Latent gammaherpesvirus can also be associated with malignancies, both lymphoid and epithelial (de-The *et al*, 1982, Fleckenstein *et al*, 1982, Chang, Y., *et al* 1994 & Cesarman, E., *et al* 1995a) and infection of B and T lymphocytes by EBV and HVS, respectively, *in vitro*, gives rise to transformed / immortalised cell lines (Pope *et al*,

Table 1.4* **The lymphocyte tropism, and diseases associated with the different gammaherpesviruses.**

Gammaherpesvirus	Host species	Lymphocyte tropism	Diseases Association
γ–1 herpesviruses			
EBV	Human	B-cell	Infectious mononucleosis B (& T) cell lymphomas Epithelial carcinomas
HV pan	Chimpanzee	B-cell	
HV pongo	Orang-utan	B-cell	
HV gorilla	Gorilla	B-cell	
HV papio	Baboon	B-cell	
CHV 14	Rhesus monkey		
CHV 15	African green monkey		
γ–2 herpesviruses			
KSHV	Human	B-cell	Kaposi's sarcoma B-cell lymphomas
HVS	Squirrel monkey	T-cell	T-cell lymphomas in non-host new world monkeys
HV ateles	Spider monkey	T-cell	T-cell lymphomas in non-host new world monkeys
HV aotus 2	Owl monkeys	T-cell	
OHV 2	Sheep	T-cell	Malignant catarrhal fever in deer
HiHV1	Roan antelope	T-cells	
AHV1	Wildebeest	T-cell	Malignant catarrhal fever in European cattle
AHV2	Hartebeest	T-cell	
BHV4	Cow		
EHV2	Horse	B-cell #	IM-like symptoms
EHV5	Horse	B-cell #	IM-like symptoms
HV sylvilagus	Rabbit	B & T cell	lymphomas
HV marmota 1	Woodchuck		
MHV-68	Mouse	B-cell	B-cell lymphomas + IM-like symptoms

Table adapted from Roizman et al,, 1995, Kieff et al, 1995, Baer et al, 1984, Bocker et al, 1980, Rabin et al, 1978, Rabin et al, 1980, Ohno et al, 1979, Neubauer et al, 1979, Gerber et al, 1977, Russo et al, 1996, Albrecht et al, 1992, Fleckenstein et al, 1982, Bridgen et al, 1989, Reid et al, 1989, Reid et al, 1991, Li et al, 1995, Lomonte et al, 1996, Telford et al, 1993, Medveczky et al, 1989, Kramp et al, 1985, Efstathiou et al, 1990b & Sunil-Chandra et al, 1994a. # not formally proved.

1986, Schirm *et al*, 1984 & Biesinger *et al*, 1992). Unlike alphaherpesvirus, the genes expressed during latency do not appear to be conserved between the different groups of gammaherpesviruses. Neither KSHV, MHV-68 or EHV2 encode genes with sequence homology to the main latency associated genes of either EBV or HVS.

1.2.9 **Gamma-1 herpesviruses**

The gammaherpesviruses are split into 2 groups based on their genome arrangements, although both groups (γ–1 and γ–2) both conform to the same conserved gene block order and orientation (*see Table 1.2*) (Gompels *et al*, 1988). The γ–1 herpesviruses are clearly distinct group of gammaherpesviruses, restricted to the old world primates. They all share a similar genomic arrangement, with respect to internal repeats, and form life long latent infections in the B-lymphocytes of their natural host. The γ–1 herpesvirus group includes EBV, HV gorilla, HV pan, HV papio and HV pongo, their natural hosts being man, gorilla, chimpanzee, baboon and orang-utan, respectively. The γ-1 herpesviruses can all directly transform their host's peripheral B-lymphocytes, *in vitro* and peripheral blood leukocytes (PBL), from sera positive individuals, spontaneously give rises to virally infected transformed B-cell lymphoblastoid cell lines (LCL) when cultured, *in vitro* (Dillner *et al*, 1987, Rabin *et al*, 1978, Neubaner *et al*, 1979 & Pope *et al*, 1968). They all show between 35%-45% colinear sequence homology (Heller *et al*, 1982, & 1978 & Neubaner *et al*, 1979), are antigenically similar (Rabin *et al*, 1980) and produce EBNA like proteins in association with B-cell latency (Dillner *et al*, 1987).

1.3.1 **Gamma-2 herpesviruses**

The γ–2 herpesviruses comprise a less clearly distinct group compared to the γ–1 herpesviruses. The γ–2 herpesviruses were originally defined as T-cell tropic gammaherpesviruses with a 'B' type genome structure, as typified by HVS and HV ateles. However, although many γ–2 herpesviruses do conform to the 'B' type genome structure, EHV-2 has an 'A' and EHV-5 has a 'F' type genome structure.

They are not universally T-cell tropic since KSHV and MHV-68 have been shown to be B-cell and not T-cell tropic. An alternative definition of the γ–2 herpesvirus sub-group would be any gammaherpesvirus, be it human, cebine, bovine, equine, ovine, or murine, that is not specifically a γ–1 herpesviruses.

1.3.2 **Sub-groups within the gamma-2 herpesviruses**

The γ–2 herpesviruses is essentially comprised of a number of γ–2-sub-groups. These comprise of the 'HVS-like' gammaherpesviruses of the new world monkeys. These form a distinct group analogous to the 'EBV-like' γ–1 herpesviruses. The recently isolated KSHV is probably a human form of a 'KSHV-like' γ–2-sub-group that infect old world monkeys (Rose *et al*, 1997) and is genetically closely related the 'HVS-like' γ–2-sub-group (Russo *et al*, 1996).

The MCF inducing 'AHV1-like' gammaherpesviruses also probably form a distinct γ–2 sub-group that infect ruminants. These include AHV1, AHV2, ovine herpesvirus (OHV) 2 and Himalain herpesvirus (HiHV) 1, the sub-group does not include BHV4. The 'AHV-1-like' gammaherpesviruses have many biological properties similar to HVS. AHV-1 infection of T-cells gives rise to cell lines that will proliferate indefinitely in the presence of interleukine (IL)-2, *in vitro* and infection of a non-host ruminant can give rise to MCF, a lethal, viral initiated/driven lymphoproliferative / autoimmune disorder.

The classification of other γ–2 herpesviruses is less clear cut. EHV-2 and -5, despite having different genomic architecture, also probable represent a distinct γ–2-sub-group. Despite being antigenically distinct from one another, they have greater genetic similarity to each other than to any other gammaherpesvirus. Partial sequencing of conserved areas of the EHV-2 and -5 genome, revealed them to be only marginally more related to HVS than they are to EBV and that EHV, HVS and EBV were essentially all equally genetically divergent (Telford *et al*, 1993). Although EHV-2 and -5 encode genes, homologues of which are only found in γ–2

herpesviruses, such as a G protein-coupled receptor (GCR), encoded by HVS ORF74, they also encode a gene homologous to the EBV BCRF1 gene. This further compounds the ambiguity in their classification since the EBV BCRF1 gene is considered to be unique to the γ–1 herpesviruses.

MHV-68 may well represent a distinct γ–2-sub-group that infect rodents. MHV-68 was isolated as one of 5 antigenically related rodent isolates, MHV-60, 68, 72, 76 and 78 (Svobodova *et al*, 1982b), of which, other than MHV-68, only MHV-72 has been studied (Mistrikova *et al*, 1993 & Reichel *et al*, 1994). Based on sequencing data MHV-68 is more closely related to HVS than it is to EBV and contains a number of γ–2-spesific gene homologues. However, MHV-68 also encodes a glycoprotein (ORFM7) which shows greater homology to EBV gp340/220, considered unique to the γ–1 herpesviruses, than the positional homologue of HVS (Stewart *et al*, 1996). The genomic positioning of the MHV-68 encoded Bcl-2 homologue is also more similar to that of EBV than to HVS or KSHV. The MHV-68 encoded TK homologue has also a greater functionally similarity to the EBV TK homologue, with respect to anti-viral activation, than to the HVS TK homologue (Sunil-Chandra *et al*, 1993).

1.3.3 **Epstein-Barr virus**

Of all the gammaherpesviruses, EBV has been studied the most, since it was, until recently, the only known human gammaherpesvirus and is responsible for human disease. EBV has a 172kbp genome with a 60% G+C content. The coding quasi region is flanked by 0.5kbp terminal direct repeats (TR) and contains a set of reiterated 3kbp internal direct repeats (IR) which divide it into a long unique region (LUR) and a short unique region (SUR). A group of 3 small internal repeats further sub-divide the LUR sequence into a set of unique regions named U2-U5, with U1 being SUR. With the exception of the TR sequences, EBV shows a high degree of co-linear homology with the other γ–1 herpesviruses (Heller *et al*, 1981a, 1981b & 1982).

1.3.4 **EBV Genome and genetics**

EBV was the first herpesvirus to be cloned and sequenced (Dambaugh *et al*, 1980, Baer *et al*, 1984, Hatfull *et al*, 1988 & Parker *et al*, 1990). EBV codes for the full compliment of 'core' herpesvirus gene products as well as the partially conserved herpesvirus genes (*see Tables 1.2 and 1.3*). As well as the core genes EBV also encodes 18 genes which are seemingly unique to the gammaherpesvirus. Of these genes, BRLF1 codes for the EBV R-transactivator and BHRF1 codes for a functionally active Bcl-2 homologue (Henderson *et al*, 1993 & Tarodi *et al*, 1994). The remaining genes encoded by EBV are unique to the either the γ1 herpesviruses or to EBV specifically. These include the BZLF1 gene, the BCRF1 gene and all the genes which code for the latency associated proteins. The BZLF1 gene codes for the protein associated with reactivation from latency, named the 'Z fragment EBV reactivation antigen' (ZEBRA), and is believed to be closely related to the cellular fos/jun family of proteins (Packham *et al*, 1990). As well as inducing the reactivation of the latent EBV genome, ZEBRA also causes the induction of cellular genes. Expression of ZEBRA has been shown to up regulate the expression of tumour growth factor (TGF)-β, which is a powerful immunosuppressing cytokine (Cayrol *et al*,1995) . The BCRF1 gene codes for a cellular IL-10 homologue (Moore *et al*, 1990 & 1992). This is a gene expressed during lytic replication as opposed to during latency (Stewart *et al*, 1992 and 1994) and has been shown to increase the efficiency by which EBV can infect / transform B-cells *in vitro* (Stuart *et al*, 1995). BCRF1 expression may also act to partially suppress the action of CTL and NK cells, since IL-10 is a Th2 cytokine and has been shown to down regulate IL-2 and interferon (INF)-γ production by T-cells and macrophages (Stuart *et al*, 1995 & Moore *et al*, 1993). Unlike many of the γ–2 herpesviruses, EBV does not code for a GCR, cyclin D or a complement regulatory protein (CRP) homologue. However, EBV does specifically up regulate 2 cellular GCR proteins (Birkenbach *et al*, 1993), cyclin D2 (Sinclair *et al*, 1994) and complement receptor (CR) 2 (also termed CD21) (Wang *et al*,1990).

1.3.5 *In vitro* infection of B-lymphocytes and epithelial cell with EBV

Initial viral attachment to B-lymphocytes occurs through interactions between EBV gp350 and the cellular CR 2 (CD21). However, penetration of the B-cell membrane requires the three EBV glycoproteins, gH, gL, and gp42, in a complex. Cellular MHC-class II, through an interaction with gp42, acts as an essential B-cell entry associated co-factor (Li *et al*, 1997). Despite being infected *in vivo*, epithelial cells can not normally be infected *in vitro*. However, epithelial cell lines transfected with CD21 can be directly infected with EBV (Li *et al*, 1992). Viral entry into the cell is mediated via a different mechanism than with B-cells, since epithelial cells do not express MHC class II. Monoclonal antibodies that bind gp42 block virus entry into B-cells but not CD21 transfected epithelial cell lines. Moreover, monoclonal antibodies that specifically bind the gL/gH complex will block virus entry into CD21 transfected epithelial cell lines but not B-cells (Li *et al*, 1995).

Upon infection of B-lymphocytes, the viral genome circularises to form a covalently closed episome. Transcription initially starts from a promoter at the SUR end of the IR1 sequence called the BamW promoter (Wp). The transcript undergoes multiple splicing events. This can result in the generation of an ATG translation start site, by frame shift, leading to the expression of the EBNA leader protein (EBNA LP), which is coded for at the start of the U2 region. Alternatively, if splicing fails to generate the EBNA LP AUG, EBNA-2, which is coded for down stream of EBNA LP, gets expressed. EBNA-2 then transactivates the main EBNA transcriptional promoter, the Bam C promoter (Cp), located in the U1 region. Multiple splicing of the transcripts, initiated from Cp, allows the further expression of EBNA LP and 2. Alternatively, EBNA 3a, 3b and 3c, with are coded sequentially along the U3 region, and EBNA-1, which is coded for by an ORF spanning the IR3 region, can also be expressed. EBNA-2 also transactivates the latent membrane proteins (LMP)-1, -2a and -2b. LMP-1 is coded for on the opposite, overlapping strand to LMP-2a and -2b at the TR end of U5. LMP-2a and -2b are generated by alternative splicing events

and the transcript spans the TR region ending in U1. Also located in the U1 region are the two short non-polyadenylated RNA genes, Epstein-Barr encoded RNA (EBER)-1 & -2 and ori P, located respectively, between the LMP2 ORFs and the IR1 region. The EBERs both contain a RNA polymerase III promoter region. RNA polymerase III is believed to dominate the EBER transcription, however RNA polymerase II also plays a role (Keiff *et al*, 1996).

1.3.6 **EBV transformed lymphoblastoid cell lines**

Infection of primate B-lymphocytes with EBV, *in vitro*, leads to the establishment of a latent viral infection and transformation of the B-cell (Pope *et al*, 1968). EBV will transform progenitor and pre-B-cells as well as mature B-cells (Ernberg *et al*, 1987) and B-cell lines, infected with EBV, continue to differentiate and isotype switch in the presence of IL-4 (Jabara *et al*, 1990 & Gauchat *et al*, 1992). Based on their transforming efficiencies EBV isolates can be categorised in to two distinct groups. These were originally designated type A and B, with type A having a much greater transforming ability than type B. This has since been revised to Types 1 and 2. EBV-1 is almost genetically identical to EBV-2 except for alterations in the EBNA LP, 2, 3A, 3B and 3C genes. EBV-2 encoded EBNA-2, -3A, -3B and -3C differ from those encoded by EBV-1 by 47%, 16%, 20%, 28%, respectively (Addinger *et al*, 1985 & Semple *et al*, 1990).

After infection, the single circularised EBV genome increases in copy number for 1 to 2 weeks finally reaching a constant level at about 50 copies per cell. Transformed colonies, comprising of about 100 - 1000 cells, appear after approximately 21 days. (For reviews of EBV transformation of B-cells, see Pope *et al*, 1986, Ring *et al*, 1994 and Sugden *et al*, 1994). EBNA-1 is the only transactivating factor required for episomal maintenance. EBNA-1 binds two sites at the latent cycle origin of replication (ori P). From here it enhances it's own transcription, along with the other latent transcripts, by acting as an enhancer to the type 3 latency specific, transcriptional promoter. EBNA-2 and -3c are also transcriptional activators, with

differences in the EBNA-2 gene being largely responsible for the different transforming efficiencies of EBV type 1 & 2 (Rickinson *et al*, 1987).

EBNA-2 transactivates LMP-1 expression and with it, up regulates the B-cell activation marker CD23. From all the EBV latent gene products LMP-1 has the most transforming properties. Although LMP-1 expression alone is insufficient to transform B-cells, it can transform immortal rodent fibroblast lines. LMP-1 expression in these cells, greatly reduces their levels of contact inhibition, anchorage dependence and allows them to grow in soft agar (Baichwal *et al*, 1988). LMP-1 expression in LCL and in BL cell lines, when artificially up regulated, increases levels of homotypic adhesion molecules, such as ICAM1, LFA1 and LFA3. This greatly increases their immunogenecity to T-lymphocytes. (The properties of the EBV latent gene products, is review by Ring *et al*, 1994). LCL cell lines are 'immortal', as they replicate indefinitely , *in vitro*, never reaching crisis. They are also 'transformed', since they form tumours when grafted into an immunocompromised host. However, they are not 'malignant', since they do not form tumours in non-immunocompromised syngeneic graft recipients.

1.3.7 **EBV associated latency strategies**

Latent EBV can take 1 of 3 forms, *in vitro* (Kerr *et al*, 1992), all 3 involve different latent gene expression and are controlled by 2 different viral promoters. All 3 latency patterns, exist in cells, *in vivo* as well as *in vitro* and result in different cell phenotypes, though the three classic forms of latency only tend to be observed in conjunction with neoplastic cells which may not represent latency in 'normal' tissue / cells. All 3 types of latency can be artificially generated, *in vitro*, using EBV infected somatic cell hybrids (Kerr *et al*, 1992).

1.3.8 **EBV latency type 3**

With latency type 3, the full range of latency associated, and restricted, viral gene

expression is observed. These comprise of the 6 EBNA genes, -1, -2, -3a (-3), -3b (-4), -3c (-6) & LP, 3 LMP genes, -1, -2a & -2b, and 2 EBER species. Type 3 latency is generally associated with cells of a lymphoblastoid, transformed phenotype, of B-cell origin. LCLs can be generated *in vitro*, by culturing PBLs, from EBV sera positive individuals or by directly infecting them with EBV (Pope *et al*, 1968). After infection, the first latent transcripts detectable are EBNA-2 and EBNA-LP. These are followed by the other EBNA transcripts, then LMP-1 and finally after 70 hours post infection LMP-2a & -2b. All the latency associated transcripts have been shown to be necessary for immortalisation and out growth of *in vitro* infected B-cell, except for EBNA-3c, LMP-2a & -2b and the EBERs. Type 3 latency is associated with, largely non-malignant, polyclonal, LPD in immunosuppressed transplant patients (Cleary *et al*, 1986) and AIDS patients (Hamilton-Dutoit *et al*, 1993). Similar LPD occurs in experimental EBV infections of cotton-top tamarins (*Somguinus Oedipus oedipus*) (Cleary *et al*, 1985), and with the adoptive transfer of PBLs, from EBV sera positive donors, or LCLs into severely immunocompromised inbred (SCID) mice strains (Purtilo *et al*, 1991 & Rowe *et al*, 1991).

1.3.9 **EBV latency type 1**

Type 1 latency is associated with EBV positive BL cells, *in vivo*, and BL cell lines that have retained their BL phenotype, *in vitro* (Rowe *et al*, 1987). Type 1 latency is also commonly associated with AIDS related, malignant "Burkitt's like" lymphomas (Hamilton-Dutoit *et al*, 1993). EBV latent gene expression is restricted to EBNA-1 and the EBERs. The latency transcriptional promoter, used in both type 1 and 2 latency, the Bam F promoter (Fp) is different to the one used in type 3 latency (Kerr 1992). EBNA-1 negatively feeds back on Fp promoted transcription, by binding a low affinity site, in the Q locus, 200bp down stream of the Fp transcriptional start site. In this way the episomal copy number per cell can be maintained at a constant level without over production of EBNA-1 in non-replicating cells (Sung *et al*, 1994). This is believed to play a critical role in the prevention of host clearance of latently infected B-cells, in healthy, sera-positive individuals. When cells infected with

EBV, in type 1 latency, start to under go cell division, the cell cycle specific transactivator, E2F, displaces EBNA-1 from the Q locus so allowing Fp driven EBNA-1 expression to occur again (Sung *et al*, 1994). This may explain why none of the three 'classical' forms of latent gene expression are observed in the resting pool of latently infected B-cells in normal healthy EBV sera positive individuals. Latent virus is restricted to resting B-cells which are CD19 positive but CD80 (B7) negative. The latent viral gene expression is restricted to only the EBERs and LMP2A (Miyashita *et al*, 1995 & Decker *et al*, 1996). Because resting B-cells do not generally replicate, there is no need for EBNA-1 expression. Expression of LMP2A is believed to prevent the virus from reactivating by blocking stimulatory cellular transduction pathway signalling (Miller *et al*., 1995).

1.4.1 **EBV latency type 2**

Latency type 2, in many respects is similar to type 1. It is associated with EBV positive Hodgkin's disease lymphomas, undifferentiated nasophorangeal carcinoma (50%) and up to 25% of AIDS related malignant "Burkitt's like" lymphomas (Deacon *et al*, 1993, Brooks *et al*, 1992 & Hamilton-Dutoit *et al*, 1993). Type 2 latency has also been observed in cloned sub-populations of EBV infected CD21 transfected epithelial cells (Knox *et al*, 1996). Type 2 gene expression consists of EBNA-1, LMP-1,-2a & -2b and the EBERs. Given that LMP-1 can induce morphological transformation in human keratinocytes, inhibit differentiation in epithelial cells and is associated with the development of hyperplastic dermatitis, in transgenic mice (Ring *et al*, 1994), it is possible that it's expression may play a role in the development of these malignancies.

1.4.2 **Reactivation of EBV from latency**

It has been hypothesised that spontaneous changes in latency, in EBV infected B-cells, may account for the fact that healthy sera positive individuals never completely eliminate EBV from their B-cell population and the levels of latent gene product-

specific, memory T-cells, remain high throughout an infected individuals life. Though type 3 latently infected cell may be able to directly give rise to latency type 1 cells only the converse has only been shown to occur, using *in vitro* BL derived cell lines, that loose there BL like phenotype (Rowe *et al*, 1987). Infected B-cells derived from healthy sera positive individuals, will spontaneously give rise to LCL cell lines, which contain EBV in a type 3 latent state, *in vitro* (Rickinson *et al*, 1986), but to achieve this they have to reactivate and infect surrounding B-cells. This may well represent the *in vivo* situation since BZLF1, the gene responsible for viral reactivation, expression can be detected in B-cells during IM along with late viral gene expression (Schwarzmann *et al*, 1996).

Stable EBV latency, in B-cells, can be disrupted by a number of chemical reagents *in vitro* and reactivation is believed to occur spontaneously, *in vivo*, in the oropharynx, to allow sporadic shedding of infectious virus. A number of chemicals, such as Iodo-2'-deoxyuridine (IUdR) and n-Butyric acid (n-BA), as well as phorbol esters, such as 12-O- tetradecanoylphorbol 13-acetate (TPA) can induce a low level productive infection in EBV positive B-cells, *in vitro* (Bauer *et al*, 1982 & Hansen *et al*, 1978). These chemicals are highly toxic and cannot be used *in vivo*. Anti-immunoglobulin, crosslinking antibodies, serum factors and bryostatin 1 have also been shown to induce productive replication in latently infected cell lines, *in vitro* (Bauer *et al*, 1982 & Stewart *et al*, 1993). The viral gene product responsible for the switch, from latent infection to productive infection, is encoded by the BZLF1 gene and has been termed the Z fragment Epstein-Barr reactivator (ZEBRA) protein (Countryman *et al*, 1987). The re-seeding of productive virus to the oropharynx, to allow sporadic viral replication in the epithelial cells and virus shedding into saliva, is believed to occur due to reactivation of latently infected B-cells, at this site. Though this is still theory, reactivation of latently infected B-cells to produce infectious virus has been shown to occur *in vitro* (Rickenson *et al*, 1986) and *in vivo* (Schwarzmann *et al*, 1996).

1.4.3 **EBV primary infection and associated diseases**

Primary infection with EBV during early childhood is generally considered to be asymptomatic. However, 50% of individuals, who become primarily infected post-puberty, develop IM. Primary EBV infection is believed to initiate at the oropharyngeal epithelium. From here it establishes a life long latent infection of peripheral blood B-lymphocytes. During IM, there are high viral titres, shed in the oropharynx. This is believed to be due to productive infection of the oropharyngeal epithelium. CD21 transfected epithelial cell lines infected with EBV *in vitro*, will spontaneously enter into productive virus replication (Knox *et al*, 1995).

The symptoms of IM, being malaise, fever, pharyngitis, lymphoadenopathy and splenomegaly, are thought to be a result of an over stimulation of the cellular immune system, in response to the EBV infection. The over stimulation results in a large scale, polyclonal activation of a non-specific humoral and cellular immune response. There is also a polyclonal expansion of latently infected B-lymphocytes, in the blood and lymphoid organs during IM. The expansion normally results in up to 1/100 host B-cells being EBV positive, but can be as high as 1/10. The proliferating latently infected B-cells are probably expressing the full complement of latent genes, since EBNA specific humoral responses are mounted during IM, and CTLs can be isolated which are LCL specific, by direct cytotoxicity assay (Svedmyr *et al*, 1975). EBV infected B-cells are highly immunogenic, when in this state of latency, and are probably responsible for the over stimulation of the cellular immune system, giving rise to elevated levels of atypical, thymic derived, circulating and tissue infiltrating lymphocytes. IM lasts for about 4 weeks, by which time the levels of latently infected B-lymphocytes are very greatly reduced by CTL activity. Though latently infected B-cells are never completely cleared from sera positive individuals, their numbers in peripheral blood are generally very low and any further non-malignant proliferations, are generally prevented by the hosts immune system (Rickinson *et al*, 1986).

For prolonged periods of time after the acute infection, virus is sporadically shed from the oropharynx. Although no further disease occurs, in normal EBV sera positive individuals, the infection is never cleared, and because of this, it is believed that up to 95% of the world's human population carry the virus. The number of latently infected B-cells during convalescence ranges from 10 - 500 infected B-cells per 10^7 peripheral B-lymphocytes (Khan *et al*, 1996). It was also observed that although frequencies varied quite markedly between different individuals. The variation that occurs within single individuals however, over time contributes less than 10% of the variation seen within the population as a whole.

1.4.4 **Non-malignant disease associated with immunocompromised hosts**

There are essentially 3 forms of fatal IM (FIM), all of which are very rare. The 2 standard forms are sporadic FIM, which affects both males and females, of a median age of 13 years old, and X-linked lymphoproliferative syndrome (XLP), which affects only males, of a median age of 2 years old. The third form of FIM is exclusively encountered by young EBV sera-negative, immunosuppressed, renal transplant patients, and is a fatal LPD like syndrome. FIM is commonly associated with severe splenomegaly, with 25%-40% of cases suffering splenic rupture. XLP is caused by an X-associated gene that results in an unknown form of immunodeficiency, hence the disease is generally only seen in male whose young age reflects their very high susceptibility to infection. Sporadic FIM tends to effect males and female at about the same frequency and often shows familial patterning. This has lead to the belief that it too, is a genetic based form of unknown immunodeficiency, though more subtle, in nature, than the XLP associated immunodeficiency. All 3 forms are assisted with bone marrow and lymphoid cell necrosis and are histopathologically similar (Weisenburgen *et al*, 1986).

EBV is also believed to be the causative agent in multi-focal, B-cell LPD, which is associated with EBV sera-positive transplant and AIDS patients (Cleary *et al*, 1986 & Hamilton-Dutoit *et al*, 1993a). The lymphomas associated with LPD tend to be

polyclonal, non-neoplastic and cellular replication is largely if not exclusively viral driven. This is very different from the, EBV positive, Burkitt's 'like' lymphomas, which are spontaneous in development, monoclonal and highly neoplastic, always being associated with tumour cell genetic / chromosomal alterations. EBV positive LPD has been associated with kidney, heart, heart / lung and liver transplants, as well as with patients with immunosuppressed bone marrow. Being non-neoplastic in nature, they will often regress in response to alleviation from the post transplantational immunosuppressive regimes. If the levels of immunosuppression are not reduced, transplant related LPD tends to be fatal (Cleary *et al*, 1986). LPD associated with the latter stages of AIDS are invariably fatal and commonly involve the central nervous system. Systemic immunoblast rich / large cell lymphomas are also reasonably common and are up to 80% associated with EBV. In general LPD in AIDS patients is similar to that of transplantation associated LPD, showing similar patterns of EBV latent gene expression, and cellular proliferation being essentially virus driven (Hamilton-Dutoit *et al*, 1993). Another EBV induced, AIDS related condition, is oral hairy leukoplakia (Thomas *et al*, 1991). Within the tissue, EBV continuously replicates causing tissue damage to the oral epithelium and the shedding of high titres of infectious virus into the saliva of the patient.

1.4.5 **EBV associated malignant conditions**

EBV is associated with a number of malignant conditions, one of the most prevalent being with eBL. Ninety six percent of eBL cases are EBV DNA positive, at the cellular level (de-The *et al*, 1978). Other forms of BL are also directly associated with EBV, at the cellular level. There is about a 40%-50% EBV association with AIDS related Burkitt's 'like' lymphoma (Hamilton-Dutoit *et al*, 1991a) and a 15% association with sporadic BL (sBL) (Lenier *et al*, 1986 & Toren *et al*, 1994). Other malignant conditions that are associated with EBV are undifferentiated NPC, Hodgkin's disease and peripheral T-cell lymphomas. NPC is highly associated with EBV and is the most common cancer in Chinese males and second most in females. Hodgkin's disease and peripheral T-cell lymphomas have a lower association (de-

The *et al*, 1982, Hamilton-Dutoit *et al*, 1991b, Brooks *et al*, 1992, Deacon *et al*, 1993 & Corbo *et al*, 1994). The role EBV plays in the development of malignant conditions is highly controversial, since all are associated with cellular genetic / chromosomal alterations, as well as inappropriate expression of cellular oncogenes.

1.4.6 **The roles of EBV in malignancy development**

The most common oncogenic alteration associated with BL cells is the deregulated, constitutive expression of the cellular proto-oncogene c-myc. This is normally achieved by translocation of the c-myc gene, from 8q24 to the immunoglobulin heavy chain locus 14q32, although it also can get translocated to the Ig light chain loci, 2p13 or 22q11 (Klein *et al*, 1983). Although deregulated c-myc expression can induce direct immortalisation in some experimental systems (Bernard *et al*, 1989), it cannot directly transform lymphocytes. Being latently infected with EBV, may well enhance progression to malignancy, in cells with deregulated c-myc. Deregulated c-myc expression in transgenic mice has been shown to vastly increase the probability of spontaneous B-cell lymphoma development (Leder *et al*, 1986) and EBV through it's immortalising functions can prevent the cell undergoing apoptosis in response to inappropriate proto-oncogene expression or chromosomal damage. Over expression of c-myc in EBV positive LCL cells causes an increase in susceptibility to non-MHC restricted NK and lyphokine activated killer (LAK) cells, *in vitro* (Cuomo *et al*, 1993). This could explain why eBL, associated with the malaria belts of sub-Saharan Africa and New Guinea, and AIDS associated BL have a high association with EBV but sBL, which affect healthy individuals, has a low association with EBV. It is possible that EBV infected B-cells, in individuals immunocompromised through persistent infection with the malaria parasite or HIV, can acquire oncogenic mutations over a period of time and so go on to form BL (de The *et al*, 1982).

Although EBV gene BHRF1, does encode a viral proto-oncogene, which shows limited sequence homology, but good functional homology, to the apoptosis inhibitor Bcl-2 (Henderson *et al*, 1993), it is not believed to be expressed during latency.

Other EBV genes, such as EB2, are also potentially transforming (Corbo *et al*, 1994) but again do not seem to be appropriately expressed. This appears to only leave LMP1, which is only expressed in a proportion of EBV positive malignant conditions (Brooks *et al*, 1992, Deacon *et al*, 1993). It remains controversial to whether EBV plays a causative role in malignancy development or is merely a passenger. There is evidence that EBV is one of the main causes of the malignant phenotype of the BL Akata cell lines, since the EBV negative clonal cell lines derived from the same tumour as EBV positive lines, lose their malignant phenotype as compared to EBV positive clonal lines from the same origin (Shimizu *et al*, 1994).

The role of EBV may be very different during the early and late stages of tumourogenesis. EBV infected CD21 transfected immortal epithelial cell lines take on a more transformed phenotype. When injected subcutaneously into nude mice with TPA the EBV cells developed into poorly differentiated carcinomas (Li *et al*, 1997). However, in NPC, EBV shows a highly restricted pattern of gene expression. Similar patterns of EBV gene expression have been observed in sub populations of EBV infected CD21 transfected epithelial cells which show a lower degree of differentiation (Knox *et al*, 1996).

1.4.7 **Herpesvirus saimiri**

HVS is a member of a group of oncogenic, T-cell tropic γ–2 herpesviruses, which also includes HV ateles and HV aotus type 2, the natural hosts being new world primates. HVS infection of its natural host, the squirrel monkey (*Saimiri sciureus*), is asymptomatic (Falk *et al*, 1972) but infection of other new world monkey species and cotton tailed rabbits leads to the rapid development of highly malignant T-cell lymphomas (Fleckenstein *et al*, 1982 and Medveczky *et al*, 1989). Infection of PBLs of non-host New World monkeys results in the out growth of virally infected, transformed/immortalise T-cell lines (Schirm *et al*, 1984 & Biesinger *et al*, 1992). The resultant T-cell lines can be either IL-2 dependant, but lack the need for antigenic stimulation, or IL-2 independent, depending on the HVS strain used to

transform (Szomolanyi *et al*, 1987). HVS isolates can be categorised into three strains, A - C, which denotes their relative oncogenic virulence (Medveczky *et al*, 1984). HVS will transform/immortalise both α/β CD4 positive and CD8 positive T-cells, *in vitro* (Berend *et al*, 1993 & De Carli *et al*, 1993), as well as γ/δ T-cells (Yasukawa *et al*, 1995 & PachecoCastro *et al*, 1996). Transformed T-cell lines retain many of the untransformed phenotypes and specificity. CD4 positive T-cells retain there Th1 or Th2 cytokine production profiles after transformation (De Carli *et al*, 1993) and CD8 positive T-cells retain their peptide specific cytotoxicity and cytokine production profiles (Berend *et al*, 1993 & Mackewicz *et al*, 1997).

1.4.8 **HVS genome and genetics**

As with other γ–2 herpesviruses, the HSV genome consists of a unique coding region (UCR), with a low G+C content flanked at both ends by tandem repeats with a high G+C content. The tandem repeats are each 1,444 bp and the UCR 112,930 bp. The UCR codes for 75 ORFs, 38 of which code for the conserved herpesvirus "core" genes (*see Table 1.2*). Of the remaining 37 ORFs, 22 are homologous to EBV genes, 7 are γ–2 herpesvirus specific (ORFs -2, -4, & -71 to -74) and 8 are unique to HVS (ORFs -1, -3, -5, -12 to -15,& -51) (Albrecht *et al*, 1992).

1.4.9 **Gammaherpesvirus specific genes encoded by HVS**

Of the 22 non-core ORFs that HVS shares with EBV, 2 are common to both alpha and gammaherpesviruses. These ORFs code for the small unit of ribonucleotide reductase (HVS ORF60 & EBV BARF1) and the nucleotide scavenging enzyme thymidine kinase (HVS ORF21 & EBV BXLF1). The HVS TK is predicted to contain 527 residues, with the nucleotide binding domain motif located at residues 212 to 224. This region is highly conserved in the TK homologues coded by other gammaherpesviruses such as EBV, alphaherpesviruses such as HVS, VZV, MDV, HVT, the poxviruses and the cellular gene. Outside this motif the HVS nucleotide sequence and predicted peptide amino-acid sequence is far more divergent, though

retaining significant homology with the EBV gene (Honess *et al*, 1989). A further 4 genes are common to both gamma and betaherpesviruses, of which, 3 (HVS ORFs 24, 31 & 35 and EBV BcRF1, BDLF4 & BGLF3.5 respectively) are located in or close to the conserved gene block VI and the last (HVS ORF18 & EBV BVRF1.5) in block 4. The function of these genes is presently unknown (Gompels, *et al*, 1995).

Of the remaining 16 ORFs, all of which are unique to the gammaherpesviruses, only 5 code for a protein with a defined function. ORF16 (EBV BHRF1) codes for a Bcl-2 homologue, ORF40 (EBV BBLF1) codes for a helicase / primase enzyme, ORF50 (EBV BRLF1) codes for a homologue of the EBV R-transactivator, ORF65 (EBV BFRF3) codes for a capsid protein and ORF75 (EBV BNRF1) which codes for a putative tegument protein. ORF75 has a high level of homology with the *Escherichia coli* and *Drosophila melanogaster* purine biosynthesis enzyme *N*-formylglycinamide ribotide amidotransferase (FGARAT). The Bcl-2 homologue encoded by ORF16 is functionally active at inhibiting apoptosis. It contains a highly conserved SH1 and SH2 domain but not the SH3. The ORF16 product has been shown to heterodimerise with other members of the Bcl-2 family, such as BAX and BAK (Nava *et al*, 1997). ORF50 codes for two transcripts, the first has a transcriptional initiation start site located in ORF49 and is spliced, the second has a transcriptional initiation start located in the first exon of ORF50 and is unspliced. The spliced transcript codes for a transactivator which is expressed early in the lytic cycle. The unspliced transcript codes for a transactivator which is expressed much later in the lytic cycle. The two transcripts, both homologous to the EBV R-transactivator, produce products which have different affinities and transcriptional activities and are expressed in different phases of the productive replicate cycle (Nicholas *et al*, 1991 & Whitehouse *et al*, 1997).

1.5.1 **Gamma-2 herpesvirus specific genes encoded by HVS**

Of the seven γ–2 herpesviruses specific genes encoded by HVS, most code for homologues of cellular genes. ORF 2 and 70 code for enzymes involved in

nucleotide metabolism, dihydrofolate reductase (DHFR) and thymidine synthase (TS). They have an 83% and a 66% respective amino acid identity with their human cellular counterparts (Trimble *et al*, 1988 & Bodemer *et al*, 1986) and are expressed early in the lytic cycle (Nicholas *et al*, 1990). ORF4 codes for one of two CRPs encoded by HVS. The sequence of ORF4 has a potential coding capacity for 360 amino acid protein with seven sites for N-linked glycosylations, a 20 amino acid signalling domain and a C-terminal 23 amino acid transmembrane domain. Sequence comparisons have shown it to have significant homology with known members of the complement protein family that interact with C3b and C4b. These include C4b-binding protein, membrane co-factor protein (CD46) decay-accelerating factor (CD55), complement receptors CR1 (CD35) and CR2 (CD21), factor H and a 35-kDa secretory protein encoded by vaccinia virus, that has been shown to bind C4b and down regulate both classical and alternative complement pathways (Kotwal *et al*, 1988 & 1990 and Isaacs *et al*, 1992). ORF4 produces two mRNAs, the first, a 1.7kb, non-spliced mRNA that codes for a membrane glycoprotein and the second, a 1.5kb mRNA with the transmembrane coding domain removed by splicing. Both forms have been shown to confer resistance to complement-mediated lysis and to inhibited C3 convertase activity. The spliced form is probably a secretory version of the unspliced form, which is probably a membrane protein. Both are expressed during the lytic infection *in vitro* (Albrecht *et al*, 1992 & Fodor *et al*, 1995). The final two γ–2 herpesvirus specific ORFs (72 & 74) encode cell cycle regulatory molecules. ORF72 encodes a homologue to a type D cyclin, exhibiting greatest amino acid identity (25%) to the human cyclin D1 (Xiong *et al*, 1991). ORF74 encodes a GCR homologue with a 30% amino acid identity to the human interleukin (IL)-8 receptor (Murphy *et al*, 1991 and Nicholas *et al*, 1992). GCR homologues are also encoded by betaherpesviruses, such as HCMV and HHV6 (Gompels, *et al*, 1995). HVS ORF74 shares a 22% amino acid identity with HCMV US28. Both cellular cyclin D and GCR family members have been directly linked with oncogenicity and malignancy development (Jackson *et al*, 1988 & Motokura *et al*, 1991).

1.5.2 **Genes Unique to HVS**

Of the 9 ORFs that are unique to HVS, two code for homologues of cellular genes and a third for a homologue of the mouse mammary tumour virus 7 superantigen. ORF13 encodes a homologue to cellular IL-17 which exhibits a 72% amino acid identity with human IL-7 and 57% identity to the murine CTLA8. The ORF13 product, termed vIL-17 will stimulate NF-kB transcriptional activity and IL-6 secretion in fibroblasts, and co-stimulate T cell proliferation, *in vitro* (Yao *et al*, 1995a & Yao *et al*, 1995b). The ORF14 product is a secreted glycoprotein that will bind to heterodimeric MHC class II HLA-DR molecules and will act as a superantigen. ORF14 has a predicted 22% amino acid identity to the mouse mammary tumour virus 7 superantigen and will induced the proliferation of human PBMC and purifies blood T cells, *in vitro* (Yao *et al*, 1996). ORF 15 is the second of the CRP like ORFs encoded by HVS. ORF15 has a 69% amino acid identity to squirrel monkey CD59 and a 48% identity to human CD59. Cellular CD59 is a phosphatidyl-inositol-glycan-anchored glycoprotein involved in the restriction of complement-mediated lysis and T-cell activation. The HVS-CD59 has been shown to inhibit complement lysis, after C3b deposition, as effectively as its human and squirrel monkey homologues but with a greater species diversity. ORF15 expression can be detected during lytic but not latent infections (Albrecht *et al*, 1992 and Rother *et al*, 1994).

1.5.3 **HVS latency and transformation**

Cell lines derived from HVS (and HVateles) induced lymphomas, from both marmosets and cotton tail rabbits, as well as *in vitro* transformed T-cell lines, contain covalently closed circular viral genomic DNA (Kaschaka-Dierich *et al* 1982, & Szomolanyi *et al*, 1987). The origin of plasmid replication (oriP) that allows stable episomal replication is located within a 1.955-Kbp DNA fragment at the left hand end of the UCR. The 1.955-kb viral fragment includes a dyad symmetry region located between two small nuclear U-RNA (HSUR) genes and is located upstream of

the DHFR gene homologue (ORF2). Episomal replication requires a HVS encoded trans-acting factor(s), analogous in function to the EBV gene product EBNA-1 (Kung *et al*, 1996). Viral gene expression in latently infected T-cells is restricted to the HSURs, which do not code for proteins, tyrosine kinase interacting protein (Tip) and the saimiri transformation-associated protein (Stp) (Medveczsky *et al*, 1993, Lund *et al*, 1995 & Biesinger *et al*, 1995). All are encoded within the highly heterogeneous, left hand terminal region of the HVS UCR. Genetic variation in this region accounts for the differences in oncogenic virulence of the HVS isolates (Medveczky *et al*, 1984).

HVS encodes up to 7 small HSURs, which comprise the majority of viral specific transcripts within latently infected T-cells. HSURs 1, 2 and 5, which exhibit repeat sequences at their 5' ends, identical to the AUUUA motif that targets a number of proto-oncogene, cytokine, and lymphokine mRNAs for rapid degradation, are encoded between ORF1 and ORF2. The other HSURs (3, 4, 6 and 7) are encoded between ORF2 and ORF3 (Albrecht *et al*, 1992). In transformed marmoset T-cell lines HSURs 1, 2 and 5 have been shown to associate with a 32 kDa cellular protein associated with RNA degradation. Similar observations have been reported in HVS infected human T-cell lines (Myer *et al*, 1992 & Geck *et al*, 1994).

1.5.4 **Expression of the HVS transformation associated protein Stp**

Both subgroup A and C HVS strains are oncogenic in new world monkeys and will transform marmoset T-cells, in an IL-2 independent manner, *in vitro*. Subgroup C strains, having greater oncogenicity than subgroup A, will also transform human, rhesus monkey and cotton-tailed rabbit T-cells (Medveczky *et al*, 1984 & Biesinger *et al*, 1992). For both subgroups, A and C, expression of Stp is essential for both transformation of T-cells and the development of T-cell malignancies (Desrosier *et al*,1985, Murphy *et al*, 1989, Jung *et al*, 1991 and Jung & Desrosier 1991). Stp is a 19-22kDa protein (Lund *et al*, 1996) with a 17 amino acid amino-terminal domain, a central region containing 18 collagen-like repeats and a carboxyl terminal

hydrophobic transmembrane domain. The N-terminal domain contains 3 glutamic acid residues and has an overall negative charge which have been shown to be essential for the transforming abilities of Stp. Disruption of the collagen-like repeats also disrupts the transforming activity of Stp and the C-terminal hydrophobic domain is essential for membrane localisation (Jung *et al*, 1994). Both Stp -A & -C have been demonstrated as being oncogenic, independent of the viral genome. Transgenic mice that constitutively expressed either Stp-A or Stp-C develop either T-cell malignancies or epithelial malignancies, respectively (Murphy *et al*, 1994 & Kretschmer *et al*, 1996). Further more Stp-C can transform rat-1 cells *in vitro*, so they can grow in soft agar and form invasive tumours in nude mice (Jung *et al*, 1991). Although the sequence of Stp is quite variable between the different HVS strains all contain a highly conserved YAEV/I motif preceded by a negatively charged glutamic acid residue. This matches the consensus sequence for binding the SH2 domain of the src family kinases. Stp-A has been shown to bind cellular src in virally transformed T-cell lines and will act as an *in vitro* substrate for src kinase activity. After phosphorylation by src, Stp will then go on to bind both P56lck and Fyn *in vitro* (*et al*, 1997). Stp-C has also been shown to associate with the cellular proto-oncogene ras in transformed T-cell lines. Expression of Stp-C has been to activate the ras signalling pathway in cell lines, *in vitro* (Jung *et al*, 1995)

1.5.5 **HVS expression of the tyrosine kinase interacting protein Tip**

With the subgroup C HVS strains, Tip expression has also been shown to be essential for transformation of T-cells and oncogenesis (Medveczky *et al*, 1993& Lund *et al*, 1997). Tip is a 32-40 kDa protein containing a C-terminal transmembrane domain and is associated with the plasma membrane of HVS transformed T-cells (Lund *et al*, 1995). The central region and N-terminal of Tip remains in the cytoplasm and interacts with P56lck via a src family kinase regulatory domain, amino acids 106 - 113, and a SH3 binding motif, amino acids 132 -141 (Jung *et al*, 1995a, Biesinger *et al*, 1995 & Lund *et al*, 1996). Tip, from HVS strain 488, expression in Jurkat-T-cell lines and fibroblastoid cell lines has been shown to down regulate lck activity (Jung

et al, 1995b). Tip, from HVS strain 484, expression in T-cells however, leads to a several hundred fold increase in lck activity. Further more infection of peripheral blood T-cells with HVS-484 lead to a 9 fold increase in cellular lck activity, as compared to uninfected controls and infection with Tip knock out HVS-484 lead to a significant decrease in lck activity (Lund *et al*, 1997). It has been hypothesised that the lck constitutive activation by Tip is instrumental in converting HVS infected IL-2 dependent T-cells to a IL-2 independent transformed state. This has been based on the observations that P56lck is associated with T-cell receptor and IL-2 receptor signalling (Hatakeyama *et al*, 1991 & Straus *et al*, 1992) and transgenic mice that over express P56lck develop thymic tumours (Abraham *et al*, 1991).

1.5.6 **Kaposi's sarcoma-associated herpesvirus**

To date the closest genetically related human herpesvirus to MHV-68 is the newly discovered KSHV or HHV8 . The scientific world was first alerted to the possibility of an eighth human herpesvirus when tissue samples from AIDS associated Kaposi's sarcoma were found to harbour unique 'γ-herpesvirus like' DNA sequences (Chang *et al*, 1994). Since then KSHV DNA has also be found to be detectable in primary effusion lymphomas (PEL), a specific subgroup of body cavity-based (BCBL) non-Hodgkin's lymphoma (NHL) (Cesarman *et al*, 1995a) and the DNA of 2 simian 'KSHV-like' gammaherpesviruses have been discovered in two different macaque species (Rose *et al*, 1997). Cell lines generated from PELs have shown to harbour whole viral genomes in latent episomal form (Gaidano *et al*, 1996, Said *et al*, 1996 & Cesarman *et al*, 1995b). The virus from a number of these cell lines has been reactivated to produce whole virions (Renne *et al*, 1996 & Said *et al*, 1996) and the viral DNA in one such line, apart from a 3kb region at the right hand side of the long UCR of the viral genome, has been fully sequenced (Russo *et al*, 1996).

1.5.7 **KSHV genome and genetics**

The BC-1 cell line derived KSHV genome conforms to the 'C' type structural

organisation, common to the γ–2 herpesviruses. It consists of a 140.5Kb long UCR containing 5 internal repeats, flanked by multiple copies of a G+C rich 801bp terminal repeat sequence. 81 ORFs have so far been described within the UCR, representing 79% of the 137.5 kb sequenced. 38 of which correspond to the core herpesvirus genes which exist within the conserved gene blocks. 66 ORFs, including the 39 core genes, are homologous to genes coded by HVS, though 2 of which, ORFs 2 & 70 do not lie in the same co-linear order. The remaining ORFs that do not show significant sequence homology to HVS, have been named K1 to K15. 2 of these, ORFs K3 & K5, show significant homology to the BHV-4 encoded IE1 gene. The remaining 13(K) ORFs do not show significant homology to any gene encoded by a herpesvirus sequenced to date (Russo *et al*, 1996).

As is common to gammaherpesviruses KSHV encodes a functionally active Bcl-2 homologue (ORF16) (Russo *et al*, 1996 & Nicholas *et al*, 1997). ORF16 has a conserved BH1 and BH2 domains but not the BH3 domain and will inhibit apoptosis when expressed *in vitro* (Cheng *et al*, 1997). It has been shown to heterodimerise with human Bcl-2 but not with either Bak and Bax, despite being capable of suppressing Bax toxicity (Sarid *et al*, 1997).

1.5.8 **Gamma-2 herpesvirus specific genes encoded by KSHV**

KSHV encodes a number of γ2-herpesvirus specific genes, which are not found in any sequenced γ1-herpesviruses. Other than ORFs K3 and K5, KSHV also encodes homologues to DHFR (ORF2), a CRP (ORF4), TS (ORF70), the HVS ORF71 (K13), cyclin D (ORF72), an immunogenic latent nuclear antigen (ORF73) and a GCR (ORF 74) (Russo *et al*, 1996, Cesarman *et al*, 1996 & Nicholas *et al*, 1997). The ORF72 gene product has been shown to have D type cyclin functional activity *in vitro*. As with the cellular homologue, it will complex with the cycle dependent kinase cdk6 and phosphorylate the retinoblastoma tumour suppresser protein (Rb), but unlike the cellular homologue, the ORF72/cdk6 complex will also phosphorylate the histone H1 (Li *et al*, 1997 & Goddenkent *et al*, 1997). The viral GCR homologue

has also been shown to be functionally active. When transfected into cells *in vitro* it acts as an antagonist independent signalling receptor, which drives proliferation via constitutive activation of the phosphoinositide-inositol trisphosphate-protein kinase C pathway (Arvanitakis *et al*, 1997).

1.5.9 **Gene unique to KSHV**

Unlike other sequenced herpesviruses, KSHV also encodes homologues to IL-6 (ORF K2), 2 macrophage inflammatory protein (vMIP I & II encoded by ORFs K6 & K4 respectively), an interferon regulatory factor (IRF) (ORF K9) and a 'NCAM like' adhesion molecule (ORF K14) (Russo *et al*, 1996 & Nicholas *et al*, 1997). The viral IL-6 homologue has a 62% amino acid similarity to human IL-6 and can prevent mouse myeloma cell undergoing IL-6 deprivation apoptosis (Moore *et al*, 1996).

1.6.1 **Restricted patterns of KSHV gene expression**

As is the case with EBV and HVS, KSHV gene expression both in tumours *in vivo* and tumour derived *in vitro* cell lines, appears to be highly restricted. In both cases the viral genome exists in a circular episomal form as opposed to linear (Decker *et al*, 1996, Cesarman *et al*, 1995b) inferring that the viral infection is latent as opposed to productive. In latently infected KS tissue, 2 virus specific transcripts were detectable (Zhong *et al*, 1996). The first is predicted to encode a small membrane protein; the other is an unusual polyadenylated RNA that accumulates in the nucleus to high copy number. This pattern of viral gene expression is seen in the majority of the KS cells and is believed to be synonymous with viral latency. Productive viral replication is restricted to a much smaller sub population of cells in which two other KSHV RNAs are also detectable (Staskus *et al*, 1997). These viral RNAs, one of which encoded a major viral capsid protein, were expressed to relatively low levels and believed to represent late gene expression. The unusual 1.2kb polyadenylated nuclear localising RNA (PAN-RNA), is transcribed by RNA polymerase II, lacks a trimethylguanosine cap and does not associate with polyribosomes (Sun *et al*, 1996).

It contains internal regions of extremely high sequence homology to the HSURs encoded by HSV and the 5' upstream region contained both proximal and distal sequence elements characteristic of regulatory regions of the HSURs. The continual transcription of untranslated RNA is common to herpesviruses during latent infection, but a specific role in viral infection remains unproved. Both episomal and linear viral DNA has also been associated with the viral infection of peripheral blood mononuclear cells (PBMC) (Decker *et al*, 1996). This suggests that there is a latent and a productive viral infection of PBMCs. It has also been observed that the KSHV IL-6 homologue is also specifically expressed in these cells *in vivo* (Moore *et al*, 1996).

1.6.2 **KSHV association with Kaposi's sarcoma**

KSHV is highly associated with a number of hyperplastic and neoplastic conditions, the most common being KS. There are 3 forms of KS, sporadic KS, which is a rare hyperplastic condition that occurs in Mediterranean men, AIDS-associated KS which is a virulent neoplastic condition and a common cause of death in AIDS victims and iatrogenic KS a virulent neoplastic condition that generally regresses on withdrawal of immunosuppressive treatment (Beral *et al*, 1991, Schalz *et al*, 1995 and Schalling *et al*, 1995). KSHV DNA can be commonly detected in the KS tissue in over 90% of all 3 types (Chang *et al*, 1994, Chang *et al*, 1996, Cathomas *et al*, 1996a, Dictor *et al*, 1996, Kedda *et al*, 1996 and Aluigi *et al*, 1996). KSHV transcription and DNA is detectable in both the spindle cell and the flattened neoplastic epithelial cells, but not in the surrounding normal skin epithelium (Boshoff *et al*, 1995, Staskus *et al*, 1996 & Aluigi *et al*, 1996). Following reduction in immunosuppressive treatment, elimination of KSHV DNA from both the iatrogenic KS lesion and the patients blood, within the limits of detection, directly pre-empts tumour regression, leaving a KSHV negative scar (Aluigi *et al*, 1996, Kedda *et al*, 1996 & Lock *et al*, 1997). Together the evidence indicates that KSHV is directly involved in the development of KS, and not merely an opportunistic, tumour tropic herpesvirus. The issue of whether KSHV is the only etiological agent in KS development remains

controversial since a highly transforming human papovavirus called BK virus has also been found in both sporadic and AIDS-associated KS lesions, as well as in KS derived cell lines (Monini *et al*, 1996b).

1.6.3 **KSHV association with primary effusion lymphomas**

KSHV is also highly associated with AIDS-associated and non-AIDS-associated PELs, a specific BCBL subgroup, of a mature B-cell origin (Cesarman *et al*, 1995a, Cesarman *et al*, 1996, Said *et al*, 1996, Ansari *et al*, 1996 & Gessain *et al*, 1997). The AIDS-associated are also generally co-infected with EBV (Cesarman *et al*, 1995a & Ansari *et al*, 1996), where as the non-AIDS-associated PELs tend to be excessively infected with KSHV (Said *et al*, 1996). KSHV does not seem to be associated with other AIDS-associated or non-AIDS-associated lymphomas of either T or B-cell origin (Gessain *et al*, 1997) or other EBV infected BCBLs (Cesarman *et al*, 1996). The cells from PELs can be cultured *in vitro* giving rise to a set of KSHV latently infected (Said *et al*, 1996) or KSHV / EBV latently infected, transformed cell lines (Cesarman *et al*, 1995b, Gaidano *et al*, 1996 & Miller *et al*, 1997). Virions reactivated from a latently infected cell line have been shown to infect CD19 positive B-cells (Mesri *et al*, 1996), thus confirming that KSHV is B-cell tropic as well as epithelial cell tropic.

1.6.4 **KSHV association with of multicentric Castleman's disease**

KSHV has also been shown to be associated with the development of multicentric Castleman's disease (MCD), an atypical lymphoproliferative disorder often associated with KS development. KSHV DNA can be detected in the vast majority (>90%) of AIDS-associated MCD tissue, both in patients with and without KS, but much less frequently (40%) in non AIDS-associated MCD. (Soulier *et al*, 1995). KSHV has also been implicated in numerous endothelial proliferative disorders, all be it at a reduced frequency of association. These include angiosarcoma in immunocompetent individuals, (McDonagh *et al*, 1996) and various proliferative

skin lesions in AIDS and immunosuppressed patients, including basal cell carcinomas, squamous cell carcinomas, actinic keratoses, verruca vulgaris, atypical squamous proliferations, and seborrhoeic keratosis (Rady *et al*, 1995, Chang *et al*, 1994 & Chang *et al*, 1996). However, the KSHV association with these conditions, remains contentious (Dictor *et al*, 1996 & Cathomas *et al*, 1996a) and requires further verification.

1.6.5 **KSHV transmission and epidemiology**

The epidemiology of the KSHV infection likewise, remains highly controversial and to a large extent unresolved. It has been studied using essentially 4 different parameters, KS incidence, sera conversion to latent viral antigens and the presence of viral DNA in PBMCs and semen. It has long been observed that in Western countries KS is far more prevalent in HIV positive homosexuals than in patients who acquired HIV from blood products (Beral *et al*, 1991 & Schalz *et al*, 1995). This has lead to the general theory that the disease causing agent for KS development, is predominantly sexually transmitted in Western / Developed countries. This has been further reinforced by the fact that in both HIV positive and KS patients, KSHV DNA can be detected in both semen and prostate tissue but not, with the exception of patients with pulmonary KS, in sputum or throat swabs (Lin *et al*, 1995, Monini *et al*, 1996a, Howard *et al*, 1997, Staskus *et al* 1997 & Cathomas, G., *et al* 1996b). There have also been reports of KSHV DNA being detectable in skin samples, especially in immunosuppressed individuals (Rady *et al*, 1995, Cathomas *et al*, 1996b, Monini *et al*, 1996a) though these findings have proved controversial (Dictor *et al*, 1996 & Staskus *et al*, 1997).

As well as sexual transmission vertical transmission of KSHV also appears probable since in sub-Saharan Africa there is a relative high incidence of childhood KS, where the median age of development is 4 years. These cases generally arise from children whose mothers are HIV positive and are often themselves HIV positive. In these cases KSHV was probably transmitted vertically, and since the predominant tumour

distribution suggests viral entry via the gastrointestinal mucosa, via breast milk (Ziegler *et al*, 1996).

1.6.6 **KSHV detection in human populations**

Although the evidence provided by surveys looking at the incidence of KSHV infection by sera conversion and nested-polymerase chain reaction (PCR) of both blood and semen, are often highly conflicting, the overall trends are consistent with sexual transmission being the principal means by which KSHV infection occurs (*see Table 1.5*). The general incidence of KSHV with all three detection systems was highest in AIDS patents with KS, then the KS negative AIDS patients, who were predominantly homosexual (with the exception of Uganda), and lastly were the HIV negative / KS negative controls. The serological studies (Gao *et al*, 1996 & Kedes *et al* 1996) have divided populations with respect to relative promiscuity. There results show that, with respect to KSHV incidence, HIV positive homosexuals > HIV negative homosexuals > promiscuous heterosexuals > general heterosexuals. They also show that Ugandans have a much greater general incidence that is seen in Western countries. Both show that KSHV infection directly correlates to 'presumed' promiscuity.

None of the detection systems used could detect the presence of KSHV, in individuals with KS, to the same extent that KSHV can be routinely detected in the KS lesions. Given that virus load and antibody responses are generally much higher in individuals who have developed virus specific tumours, than in normal healthy virus infected individuals, it is probably fair to assume that all the detection methods under estimate the frequency of KSHV infection in the general population. The viral load in normal healthy people is probably very low since studies following the regression of iatrogenic KS on withdrawal of immunosuppressive therapy, have shown that KSHV levels in the blood decrease to undetectable levels, by PCR (Aluigi *et al*, 1996 & Lock *et al*, 1997). Variation in detection sensitivity could explain why some studies did not find KSHV in the general population at all (Whitby

Table 1.5 * **The KSHV prevalence in HIV positive and negative human populations, as detected by serology and nested-PCR.**

Sample Tested	HIV +ve KS +ve	HIV +ve KS -ve	HIV-ve KS-ve	Reference
PBMC	52% (46)	8% (143)	0% (134)[1] 0% (26)[2]	Whitby *et al*, 1995
PBMC	35% (98)	19% (64)		Humphrey *et al*, 1996
PBMC	60% (5)		60% (5)[2] 80% (5)[3]	Decker *et al*, 1996
PBMC			7% (14)	Monini *et al*, 1996a
Semen		91% (33) 64% (33)[4]	23% (30) 0% (30)[4]	Lin *et al*, 1995
Semen			91% (33)	Monini *et al*, 1996a
Semen	20% (15)	33% (9)	0% (115)	Howard *et al*, 1997
Sera conversion	83% (46)	30% (138)[1] 35% (37)[6] 8% (144)[7]	1.5% (130)[1] 7%(107)[5] 13% (23)[6]	Kedes *et al*, 1996
Sera conversion	84% (40)[8] 75% (14)[9] 84% (18)[10]	24% (40)[8] 0% (20)[8/7] 61% (35)[10]	0% (122)[8/1] 4% (107)[9/1] 56% (47)[10]	Gao *et al*, 1996

Key: *[1] General blood donor, [2] Hospital control, [3] Immunosuppressed, [4] 1st round only PCR, [5] heterosexual STD clinic visitors, [6] homosexual STD clinic visitors, [7] Haemophyliacs / IV drug users, [8] North America, [9] Italy & [10] Uganda. The % value denotes the percentage of individuals KSHV positive by nested PCR / nuclear immunostaining of latently infected cell lines, by patients sera at a 1/160 dilution. The () value denotes sample size.*

et al 1995 & Howard *et al* 1997). Other studies have provided evidence that KSHV may be ubiquitous in the general population (Monini *et al*, 1996a & Decker *et al*, 1996). It is however, hard to conceive how over 90% of the population of a western country, could be infected by a virus completely reliant on sexual transmission. HSV-2, a highly contagious sexually transmitted herpesvirus, has a prevalence, amongst white heterosexuals, of approximately 20% in north America and as low as 8% and 20% in the British blood donor and sexually transmitted disease clinic populations, respectively (Cowan *et al*, 1996 & Duncan *et al*, 1997).

Detection of KSHV may be unreliable at present as a method of determining the prevalence of KSHV in the population at large, in terms of absolute numbers, but it has proved effective as a means of predicting whether HIV positive patients go on to develop KS. In studies carried out, 42-55% of HIV sera positive who tested positive for KSHV, went on to develop KS, as opposed to 0-9% of those who tested negative (Lin *et al* 1995 & Whitby *et al* 1995). However, the initial detection level in the latter of the 2 studies failed to diagnose 9/15 test subjects who went on to develop KS. It seems unlikely that over half of them went on to contract KSHV after being diagnoses HIV positive, if the predominant route of transmission is sexual.

1.6.7 **Murine gammaherpesvirus 68**

MHV-68 was originally isolated as 1 of 5 viral isolates, from 2 species of free living rodent, the bank vole (*clethrionomys glareolus*) and the yellow tailed mouse (*Apodemus flavicollis*), during a field study in Czechoslovakia (Blaskovic *et al*, 1980). All 5 isolates were found to be antigenically related (Svobodova *et al*, 1982b) and based of electron microscopy studies, they were classified as herpesviruses. Based on their replication in epithelial and fibroblastoid cells lines, *in vitro*, the isolates were further classified as belonging to the alphaherpesvirus subgroup (Ciamper *et al*, 1981, Svobodova *et al*, 1982a & 1982b). This classification however was called into doubt when pathogenesis studies carried out in young and new-born out-bred inbred mice, demonstrated that following intranasal or oral inoculation,

MHV-68 established latency in lymphoid tissue, predominately the spleen, as opposed to in neuronal tissue (Rajcani *et al*, 1985, Sunil-Chandra *et al*, 1992b).

1.6.8 **MHV-68 genomic architecture**

MHV-68 was isolated from the brain of *Clethrionomys glareolus*. Doubts over the correct classification of MHV-68 led to the entire genome being cloned into a library of restriction fragments. The library was then used to generate a complete restriction map of MHV-68 (Efstathiou *et al*, 1990a). The cloning and characterisation of the MHV-68 genome revealed a 118 kb central unique region, flanked by a variable number of 1.2kbp repeat units. This conforms to a type 'B' genomic arrangement (as specified by Roizman *et al*, 1990), an arrangement common to herpesviruses of the γ–2 sub-grouping. The overall size is also comparable to γ–2 herpesviruses, such as HVS and HVateles (Efstathiou *et al*, 1990a).

As well as having a type B genome arrangement, as opposed to a type C (type C being common to the γ–1 herpesviruses, such as EBV), MHV-68 also has a low G+C content of 45% (Efstathiou *et al*, 1990b). The γ–2 herpesviruses also have low G+C content; HVS and HVateles have 46% and 48% respectively. The γ–1 herpesviruses have relatively high G+C content; EBV has 80% total genome. Having the low G+C genomic content, distinguishes MHV-68 from the γ–1 herpesviruses, but having a low G+C content is by no means unique to the γ–2 herpesviruses. Alphaherpesviruses, such as VZV and MDV, and betaherpesviruses, such as HHV6 also have genomes with low G+C contents, 46%, 42% and 46% respectively (Roizman *et al*, 1990).

1.6.9 **MHV-68 genetics**

Partial sequencing of 'core' genes and there relative order and orientation proved beyond doubt that MHV-68 is a gammaherpesvirus (Efstathiou *et al*, 1990b). Since then the entire genome has been sequenced (Virgin *et al* 1997). MHV-68 encodes 75

ORFs and a further 8 small 'tRNA-like' non polyadenylated RNA molecules. All the core and semi-conserved herpesviruses genes are present, except for a gene homologous to HVS ORF 41, in block VI (*see Table 1.2 and 1.3*). Of the semi-conserved herpesvirus genes, the MHV-68 TK homologue, encoded by ORF 20, has been characterised (Pepper *et al*, 1996). MHV-68 TK contains the six conserved motifs found in all herpesvirus TK homologues and is a functionally active pyrimidine kinase. Despite having greater genetic homology to the HVS TK, MHV-68, unlike HVS, is inhibited by acyclovir (ACV) (Sunil-Chandra *et al*, 1993), implying that functionally, the MHV-68 TK is more similar to the EBV TK.

1.7.1 **Gammaherpesvirus specific genes encoded by MHV-68**

MHV-68 encodes 16 genes which are considered to be gammaherpesvirus specific. These include ORF 50, a homologue of the EBV R transactivator, and ORF M11, which codes for a Bcl-2 homologue. MHV-68 however does not appear to encode genes homologous to the HVS ORFs 28 and 65, EBV genes BDLF3 and BFRF3 respectively. The HVS ORF65 / EBV BFRF3 gene is believed to encode a capsid protein.

MHV-68 encodes 5 genes which are unique to the γ–2 herpesviruses, ORFs 4, 72, 73, 74 and K3. As is the case with HVS and KSHV, ORFs 4, 72, 73 and 74 code for a CRP homologue, a cyclin D homologue, the γ–2 specific immediate early transactivator and a GCR homologue, respectively. The MHV-68 ORF K3, as with KSHV, but unlike HVS, codes for a homologue to the BHV IE1 protein. However, MHV-68 does not code for either a TS or a DHFR homologue. This is unusual for a γ–2 herpesvirus since both HVS and KSHV encode homologues to these proteins, encoded by ORFs 2 and 70 respectively.

1.7.2 **Genes unique to MHV-68**

The remaining 13 MHV-68 ORFs, M1 to 10 and M12 to 14, with the exception of

ORF M7, do not have significant homology to any known genes encoded by herpesviruses. ORF M7 encodes a glycoprotein (gp150) of molecular weight 150 kDa, which is present in the envelope of virions. ORF M7 does not show significant homology to any known γ–2 herpesvirus gene, but has limited homology with EBV gp340/220 (Stewart *et al*, 1996). The EBV gp340/220 is situated in the EBV virion envelope and mediates attachment and entry into B-lymphocytes (Nemerow *et al*, 1989). MHV-68 gp150 may well have a similar role as EBV gp340 since gp150-specific antibodies neutralise the infectivity of MHV-68 virions. Of the remaining genes unique to MHV-68, ORF M1 has been partially characterised. ORF M1 shows homology to a family of scrinc protease inhibitors encoded by the poxviruses. ORF M1 has highest homology to the same 4 regions found to be conserved between the different poxvirus serpin homologues (Bowden *et al*, 1997, Boursnell *et al*, 1988 and Ali *et al*, 1994).

1.7.3 **MHV-68 gene expression**

The gene expression of MHV-68 following *in vitro* infection of tissue culture cell lines has been partially characterised and appears to follow a similar pattern to that observed in EBV (Mackett *et al*, 1989). It is presently unclear which viral ORFs are expressed during latency however there are eight small viral RNA species which are expressed at high levels during latent infection of splenic B-cells *in vivo*. These small RNA species are coded at the left terminal of the MHV-68 UCR. The RNA species have a sequence similar to tRNA and are theoretically capable of forming a clover structure. They contain RNA polymerase promoters, but do not appear to load with amino acids (Bowden *et al*, 1997). Other herpesviruses code for latency associated non-translated RNA species, the function of which remains unproved.

Herpesvirus gB homologues (typified by HSV-1 gB) are one of the most conserved genes within the *Herpesviridae*. MHV-68 gB shows sequence homology to the gB homologues of all sequenced herpesviruses but with special regard to the gammaherpesviruses. MHV-68 gB is also expressed, in cells infected *in vitro*, in a

gammaherpesvirus specific manner (Stewart *et al*, 1994). Unlike with the other sub-families, MHV-68 gB, HVS gB and EBV gB (gp110) exists only in the immature glycosylated form, i.e. containing, only high manose N-linked glycosylations. MHV-68 gB (with a Mr of 105,000) is analogous to the precursor form of HSV-1 gB and is present only associated with the ER and nuclear membrane, not with the Golgi apparatus or plasma membrane. MHV-68 gB is also not detectable in virus particles (Stewart *et al*, 1994) demonstrating both in terms of sequence and expression how highly related it is to the gB of other gammaherpesviruses (Gong *et al*, 1990).

1.7.4 **The pathogenesis of MHV-68 infection in inbred mice**

As an example of a gammaherpesvirus, which naturally infects small rodents, MHV-68 makes an ideal model for the study of gammaherpesvirus pathology in, what is presently, the most versatile experimental host species. Intra-nasal (in) inoculation of MHV-68 into 3 to 4 week old BALB/c mice, results in an acute viral infection of the lungs. During the acute infection, viral antigen can be detected, by immunostaining, in alveoli epithelial cells and mononuclear cells. The acute lung infection is resolved by 10 days post infection, by which time a latent infection of the spleen is established. Latent virus can first be detected in the spleen as early as 7 days post infection. Latent viral titres exponentially increase, peaking at 15 days post infection, then decrease to a low level (Sunil-Chandra *et al*, 1992a). Peak viral latency is generally associated with a pronounced splenomegaly. Numbers of splenic B-cells, CD4 T-cells and CD8 T-cells, all dramatically increase during the splenomegaly. The CD8 T-cell expansion is dominated by CD8 T-cells with Vβ4 T-cell receptors. This strongly suggests that MHV-68 expresses a superantigen (Tripp *et al*, 1997). The splenomegaly resolves only after viral latency has been reduced to its base level. (for review on MHV-68 infection of inbred mice see Nash *et al*, 1994).

1.7.5 **Viral latency in the spleen**

Viral latency established in the spleen predominately localises to the germinal

centres (Bowden *et al*, 1997). The splenocytes harbouring latent MHV-68 appear to be exclusively B-lymphocytes since virus can be reactivated from B-cells, but not T-cells from the spleen of infected mice (Sunil-Chandra *et al*, 1992b). Furthermore, virus cannot be detected in the spleens of intra-nasally infected, B-cell knock-out (μMT) mice by both reactivation assay and by PCR (Usherwood *et al*,1996a). As well as being B-cell tropic *in vivo*, MHV-68 will persistently infect transformed cell lines of B cell but not T-cell origin, *in vitro* (Sunil-Chandra 1993). The NS0 B-cell line, when persistently infected with MHV-68, harbours both linear and episomal viral DNA. When these lines were cultured in the presence of acyclovir, a potent inhibitor of productive herpesvirus replication, the proportion of linear viral DNA was greatly reduced, but the episomal DNA was unaffected. These results strongly suggest the establishment of viral latency.

1.7.6 **Cellular and humoral immune responses to MHV-68 infection**

CD8 T-cells play a critical role in controlling the acute lung infection. Depletion of CD8 T-cells prior to intra-nasal infection with MHV-68, results in the development of a lethal infection in BALB/c mice. The CD8 depleted mice experienced both an acute lung infection of greater severity than the undepleted controls, and a disseminated infection in which the virus spread to other organs, such as the spleen, liver, kidneys and adrenal glands (Ehtisham *et al*, 1993). The mechanism by which the CD8 T-cells exert their effect is at present, not well understood. Mice lacking in perforin, one of the main mechanisms by which CTLs and NK cells kill infected cells, efficiently control the MHV-68 infection indicating a redundant role for this mechanism of cytotoxicity (Usherwood *et al*, 1997).

CD4 T-cells also play a critical role in both the elevated levels of latently infected B-cells seen in the spleen around day 15 post infection and the development of splenomegaly. CD4 T-cells do not however appear to play a major role in controlling the acute lung infection (Ehtisham *et al*, 1993 & Usherwood *et al*, 1995). The development of splenomegaly appears to be dependant on both CD4 T-cells and

virally infected B-cells since intra-nasal infection of μMT mice as well as CD4 T-cell deficient mice fail to develop splenomegaly (Usherwood 1995 & 1996a). CD4 T-cells may have a role in the control of viral persistence in the lungs of infected animals, since MHC class II knock-out mice develop a low level chronic infection of the lungs at late times post infection (Cardin *et al*, 1996). Host antibody responses appear to play little or no role in controlling the acute lung infection. The humoral response of BALB/c mice to MHV-68, does not become detectable until after the acute lung infection has been resolved. MHV-68 antibodies only become detectable, by ELISA, in mice sera 10 to 15 days post infection, though once established remain at high titres though out the life of the mouse (Usherwood *et al*, 1996a).

1.7.7 **Cytokine responses to MHV-68 infection**

Alpha and beta interferon (INF-α/β) appear to be important in controlling the acute lung infection. Mice lacking INF-α/β receptors are killed by MHV-68, inoculated (in), in a dose dependant manner. The acute infection in these mice is more severe both in terms of longevity and lung viral titres (Nash and Dutia personal communication). The cytokine response to MHV-68 infection appears to be predominantly IL-6 and INF-γ (Sarawar *et al*, 1996). INF-γ knock out mice, do not have an obvious phenotype in response to MHV-68 infection (Sarawar *et al*, 1997). However, mice deficient in the INF-γ receptor develop chronic splenic atrophy, though show no obvious signs of disease (Dutia *et al*, 1997) (for review on the immunological features of the MHV-68 infection see Nash *et al*, 1996).

1.7.8 **Alternative routes of MHV-68 infection**

MHV-68 infections can be initiated by both intra-peritoneal (ip) and intra-venous (iv) routes of inoculation. The infections that result from these less-natural routes are different from the infection initiated by intra-nasal route. Intra-peritoneal and intra-venous inoculations result in a more severe systemic infection. A transient acute infection of the spleen occurs and the splenomegaly occurs earlier and is more

pronounced. High titres of MHV-68 inoculated (ip) into INF-γ receptor knock-out mice and μMT mice results in a lethal infection and splenic persistence, respectively (Weck *et al*, 1996). The nature of the viral persistence in the spleens of the intra-peritoneally infected INF-γ receptor knock-out mice has not been characterised.

1.7.9 **MHV-68 association with malignancies**

As with EBV, MHV-68 is associated with LPD development. Nine percent of BALB/c mice infected long-term with MHV68 (between 9 months pi to 3 years pi) go on to develop lymphoproliferative disorders. Fifty percent of these were high grade lymphomas. The lymphomas were mainly associated with the spleen and other lymphoid organs such as the mesenteric lymph node. The LPD incidence went up from 9% to 65%, over the same time scale, when the mice were immunosuppressed with cyclosporin A (CsA) early on in the course of the infection and then allowed to fully recover (Sunil-Chandra *et al*, 1984a). The lymphomas were comprised of B-cell, showing light chain restriction and CD3 positive infiltrating T lymphocytes. One such tumour gave rise to a transformed *in vitro* B-cell cell called S11. The S11 line contains both linear and episomal MHV-68 genomes and appears to harbour both lytic and latent virus (Usherwood *et al*, 1996b)

Chapter 2: Materials and Methods

2.1.1 Cell lines used

Baby hamster kidney (BHK) cells clone 21 is an immortal fibroblastoid cell line (previously described by Sunil-Chandra 1992a).

NS0 cells are a transformed B-cell myeloma cell line (previously described by Sunil-Chandra *et al*, 1993).

Mixed glial culture clone 7 (MGC7) were derived from retrovirus transformed mouse mixed glial cells. The mouse retrovirus used contained a temperature sensitive SV40 large T antigen, which transforms at permissive temperatures (35°C) but does not at non-permissive temperatures (39°C) (previously described by Terry *et al*, 1997).

Primary splenocyte cultures were derived from the spleens of BALB/c mice and comprise of mixed splenic leucocytes.

Murine lymphoma lines - Basal lymph node derived (BLN), mesenteric lymph node derived (MLN) and splenic derived (SP-) lymphoma cell lines. All were derived from mice infected with MHV-68 and immunosuppressed with CsA (Sunil-Chandra 1994a).

2.1.2 Culture reagents

Glasgow modified Eagle medium (GMEM) (GibCo BRL).
Dulbecco's modified Eagle medium (DMEM) (GibCo BRL).
RPMI 1640 (GibCo BRL)
Foetal calf serum (FCS) - (Globepharm).
New-born Calf serum (NCS) - (Harlan Sera lab).

Tryptose phosphate broth (TPB) - (Life Technologies, Paisley)

Fungizone (100X) - (Squibb) - 200μg/ml in SDW.

L-Glutamine (100X) - (Merck - BDH) - 200mM in sterile distilled water (SDW)

Penicillin (100X) - (Merck - BDH) / - 7mg/ml dissolved in SDW and filter sterilised.

Streptomycin (100X) - (Sigma) - 1mg/ml dissolved in SDW and filter sterilised.

β-mercapto-ethanol (Sigma) (100X) - 5mM in STD and filter sterilised

Trypsin / EDTA solution tissue culture grade (Life Technologies, Paisley).

Dimethyl sulfoxide (DMSO) (Merck - BDH)

Essential amino acids (50X) (Dulbecco).

Non-essential amino-acids (Dulbecco).

Trypan blue (stock) - 1% (W/V) trypan blue (Gurr) in PBS and filter sterilised.

Trypan blue (working stocks) - 1/10 dilution of working stock with sterile PBS.

Lymphoprep (Ficoll).

Phosphate buffered saline (PBS) - 80 mg/ml sodium chloride (NaCl), 2mg/ml potassium chloride (KCl), 2 mg/ml mono-potassium orthophosphate (KH2PO4), 11.05 mg/ml mono-sodium orthophosphate (Na2HPO4) (pH 7.2), sterilisation by autoclaving.

Filter sterilising was done using sterile syringes (Becton Dickinson) and a 0.2μ sterilising filters (Sartorius).

2.1.3 **Anti-virals**

ACV stock solution (GlaxoWellcome) - 2 mg/ml suspention was made up using SDW and heated to 60°C for 1 hour, under-constant agitation. The soluble ACV was then filter sterilised whilst still hot.

4'-s-EtdU stock solution (GlaxoWellcome) - 2mg/ml suspention was made up using SDW and heated to 60°C for 1 hour, under-constant agitation. The soluble ACV was then filter sterilised whilst still hot.

2.1.4 **Tissue culture growth medium**

BHK growth medium (ECT10) - GMEM with 10% NCS, 10% TPB, Penicillin/Streptomycin (70μg/ml / 10μg/ml respective final concentrations), Fungizone (2μg/ml final concentration) and L-Glutamine (2mM final concentration).

Lymphoma cell lines culture (LCLC) medium - RPMI 1640 + 10% FCS, Penicillin/Streptomycin (70μg/ml / 10μg/ml respective final concentrations), Fungizone (2μg/ml final concentration) and L-Glutamine (2mM final concentration).

NS0 cloning medium - LCLC medium with 20% filter sterilised, exhausted uninfected NSO LCLC medium.

MGC7 growth medium - DMEM with 10% FCS, 2% (V/V) non-essential amino-acids, Penicillin/Streptomycin (70μg/ml / 10μg/ml respective final concentrations) and L-Glutamine (2mM final concentration).

2.1.5 ***In vitro*** **cell line culture procedures**

Cell numbers were evaluated by counting dilutions of the cell suspention mixed 1:1 with 0.1% trypan blue, using a haemacytometer. Viability was determined by the proportion of cells that do not stain after a five minute incubation with the trypan blue.

BHK cells were grown up in T175 flasks (Nunc), at 37°C, with 50ml of ECT10 medium. Flasks were seeded with $5x10^6$ cells and cultured for 3 to 4 days, or until confluent. Confluent monolayers had the spent medium removed, washed once with PBS and incubated for 1 to 2 minutes with 5ml of trypsin/EDTA, pre-warmed to 37°C. The monolayers were separated from the bottom of the flasks by external agitation and then the trypsin quenched with 15ml ECT10. The cells were dispersed

by vigorous pipetting and pelleted by centrifugation at 1800 RPM for 5 minutes in a Beckman TJ-6 centrifuge. The supernatant was removed and the cells resuspended in 10ml of fresh medium. The cells were pelleted and resuspended in fresh medium a further two times to remove all traces of the trypsin. (previously described by Sunil-Chandra 1992a).

The NS0, MLN, BLN and SP- lymphoma cell lines were grown up in T75 (Nunc) flasks at 37°C, with 20 ml of LCLC medium. Flasks were seeded with $5x10^6$ cells and cultured for 3 to 4 days. The cell lines grow as a single cell, partial suspention culture. Weakly adherent cells were put into suspention by the vigorous use of a pasteur pipette. The cells were pelleted (1600 RPM for 5 minutes in a Beckman TJ-6 centrifuge) and resuspended in 10ml of fresh LCLC medium twice (previously described by Sunil-Chandra *et al*, 1993).

MGC7 cells were grown up in Nunc T175 flasks, at 34°C, with 50ml of culture medium. Flasks were seeded with $5x10^6$ cells and cultured for 3 to 4 days, or until confluent. Confluent monolayers had the spent medium removed, washed once with PBS and incubated for 1 to 2 minutes with 5ml of trypsin/EDTA, pre-warmed to 37°C. The monolayers were separated from the bottom of the flasks by external agitation and then the trypsin quenched with 15ml ECT10. The cells were dispersed by vigorous pipetting and pelleted by centrifugation at 1600 RPM for 5 minutes in a Beckman TJ-6 centrifuge. The supernatant was removed and the cells resuspended in 10ml of fresh medium. The cells were pelleted and resuspended in fresh medium a further two times to remove all traces of the trypsin (previously described by Terry *et al*, 1997).

2.1.6 **Storage and retrieval of cell lines from liquid nitrogen.**

Cell lines were stored frozen in liquid nitrogen. Cells were pelleted by centrifugation and resuspended in FCS, $2x10^5$ cells/ml. The cell suspention then had an equal volume of 20% (V/V) DMSO dissolved in cooled FCS and mixed. The cell

suspention was then dispensed into cryovial (Nunc) in 1.8 ml aliquots and frozen at -85°C for 24 hours. The vials were then placed in liquid nitrogen for indefinite storage.

Cell retrieved from liquid nitrogen were thawed rapidly and cold growth medium was added gradually with constant swirling. After 10ml of medium was added the cells were pelleted by centrifugation and the medium discarded. The cells were then resuspended in 10ml of fresh medium and repelleted a further two times to remove all traces of DMSO. The cells were then added with 7 ml of medium to a T25 (Nunc) flask and incubated at an appropriate temperature (37°C or 34°C) until confluent. The cells were then passaged and transferred to either a T75 or T175 depending on cell numbers or cell line used.

2.1.7 **Splenocyte preparation**

Whole spleens were mashed in a sterile stainless-steel tea strainer (John Lewis Ltd), with 4 ml of cold LCLC medium, in a 60mm petri-dish (Sterilin). The strainer was rinsed with a further 3ml of cold lymphoma cell line culture medium. Cell clumps were dissociated using a pasteur pipette and the cell suspension transferred to a universal (Sterilin). Cells were pelleted by centrifugation at 1600 RPM for 5 minutes in a Beckman TJ-6 centrifuge, set to 4°C. The supernatant was removed and the cells resuspended by external agitation and placed on ice. The red cells were removed by water lysis, 1ml of sterile distilled water added to the dissociated cell pellet and incubated for 10 seconds at room temperature, followed by 10 ml cold LCLC medium. The cells were pelleted by centrifugation at 1600 RPM for 5 minutes in a Beckman TJ-6 centrifuge, set to 4°C. The supernatant was removed and the cells resuspended by external agitation and replaced with 5ml cold LCLC medium. The cells were put into suspention using a 5ml pipette, the clumps of lysed red cells allowed to settle out, and the white cell suspention removed, put into a bijoux (Sterilin) and then placed on ice.

2.1.8 **Ficoll purification of splenic lymphocytes**

Whole spleens were mashed in a sterile stainless-steel tea strainer (John Lewis), with 4 ml of cold LCLC medium, in a 60mm petri-dish (Sterilin). The strainer was rinsed with a further 4ml of cold lymphoma cell line culture medium. Cell clumps were dissociated using a pasteur pipette and the cell suspension was overlaid onto 10ml of lymphoprep, in a universal. The lymphoprep overlays were centrifuged at 2500 RPM for 20 minutes in a Beckman TJ-6 centrifuge, set to 4°C. The lymphocytes are then removed from the medium-lymphoprep interface with a pasteur pipette and mixed with fresh medium, made up to 20ml, in a fresh universal. The cells were then pelleted by centrifugation at 2000 RPM for 10 minutes in a Beckman TJ-6 centrifuge, set to 4°C. The supernatant was removed and the cells resuspended by external agitation. The cells were resuspended in 10ml fresh LCLC medium and were repelleted and resuspended twice more, by centrifugation at 1600 RPM for 5 minutes in a Beckman TJ-6 centrifuge, set to 4°C. The cells were finally resuspended at a density of 10^7 cells per ml and put on ice.

2.1.9 **Virus stocks**

MHV-68 was originally obtained from the late Professor J. Lesso, Institute of Virology, Academy of science, Slovakia (Blaskovic *et al*, 1990). The virus stocks used though out this study were generated from the sub-master stock of clone G2.4 (Estathiou *et al*, 1990). To generate the initial MHV-68 stocks, BHK cells, $5x10^6$ per T175 flask, were infected at a multiplicity of infection (MOI) of 0.1 plaque forming units (pfu) MHV-68 per cell. The later MHV-68 working stocks were generated by infecting BHK cells at an MOI of 0.01. To allow the virus to adsorb to the cells, the inoculated BHK cells were suspension in 5ml ECT10 and then incubated for 1 hour at 37°C, on a shaker. The cells were then transferred to T175 flasks containing 50ml ECT10 and cultured for 3-4 days at 37°C. The monolayers were then removed from the flasks using a plastic cell scraper (Sterilin) and pelleted by centrifugation at 1800 RPM for 5 minutes in a Beckman TJ-6 centrifuge, set at 4°C. The cells were dance

homogenised in 2 ml / monolayer of cold ECT10 and the debris pelleted by centrifugation at 3000 RPM for 15 minutes in a Beckman TJ-6 centrifuge, set to 4°C. The supernatants were dispensed in 0.2 ml aliquots and stored at -80°C (*see Table 2.1*).

2.2.1 **4'-s-EtdU resistant virus cloning**

The polyclonal resistant virus stock was derived from the crude lysate of a persistently infected MGC7 culture, grown in medium supplemented with 2μg/ml 4'-s-EtdU. The titre of the polyclonal virus stock was established by infectious virus assay. BHK cells were infected with halving dilutions of the resistant virus from 4 pfu/10^5 cells to 0.125 pfu/10^5 cells in ECT10 supplemented with 4'-s-EtdU 2μg/ml. After a 1 hour absorption at 37°C the cells were then pipetted into 24-well plates, 10^5 cells per well. After 4 days culture, the medium from each well was removed and stored separately at -80°C. The monolayers were fixed with formyl-saline and stained with toluidine blue. The medium from the selected wells (MHV-68 positive from the dilution 0.25 pfu/well) were then used to infect cell in a similar manner, for a second and then a third round of cloning. Following the third round of cloning, six cloned isolates selected, termed mixed glial culture clone 7 derived 4'-s-EtdU resistant virus (MERV) for further characterisation (*see Table 2.1*).

2.2.2 **Infectious virus assay**

Regents

Formyl-saline - (Surgipath Europe Ltd) - 10% neutral buffered formaldehyde solution.

Toludine blue (stock)- (Merck BDH) - 1% (W/V) in distilled water.

Table 2.1 **The titres of the MHV-68 virus stocks used in this study.**

MHV-68 virus stock	Virus titre
[1]Working stock 1	10^7 pfu/ml
[1]Working stock 2	$3.5x10^7$ pfu/ml
[1]Working stock 3	10^8 pfu/ml
[1]Working stock 4	$3.5x10^8$ pfu/ml
[2]MERV (34/22/41)	$2.2 x10^7$ pfu/ml
[2]MERV (34/22/44)	$3.0 x10^7$ pfu/ml
[2]MERV (43/62/67)	$3.6 x10^7$ pfu/ml
[2]MERV (43/62/72)	$3.2 x10^7$ pfu/ml
[2]MERV (43/68/89)	$2.8 x10^7$ pfu/ml
[2]MERV (43/68/96)	$2.5 x10^7$ pfu/ml

Key: [1] Wild type MHV-68. [2] 4'-s-EtdU resistant MHV-68 variants.

Protocol

Tissue homogenates or virus samples were serially diluted and 0.2 ml was added to a bijoux containing $1x10^6$ BHK cells in 2ml ECT10. The virus was then incubated with the cells at 37°C for one hour on a shaker. A further 4 ml ECT10 was added to each bijoux and the contents of each added to a 60mm petri-dish and incubated at 37°C for 4 days. The medium was then removed and the monolayers fixed with formyl-saline and stained with toludine blue. The plaque counts were established by microscopic (Wild Heerbrugg 1274) examination of the stained monolayer. Virus titre was evaluated by multiplying the plaque counts by the dilution factors (as previously described by Sunil-Chandra *et al*, 1992b).

2.2.3 **Infectious centre assay**

Splenocyte preparations were serially diluted in lymphoma medium and 0.5ml of the resultant cell suspensions added to a bijoux containing $1x10^6$ BHK cells in 6ml LCLC medium. The contents of each bijoux was then added to a 60mm petri-dish and incubated at 37°C for 5 days. The medium was then removed and the monolayers fixed with formyl-saline and stained with trypan blue. The infectious centre counts was established by microscopic examination of the stained monolayer. The infectious centre titre was determined by multiplying the infectious centre counts by the dilution factors (as previously described by Ehtisham *et al*, 1993).

2.2.4 **Lung co-cultivation assay**

Half a mouse lung was dissected into 8 pieces and placed in a 60mm petri-dish with $1x10^6$ BHK cells and 6ml LCLC medium. The lung tissue is the cultured at 37°C for 5 days. The lung tissue is then homogenised along with the monolayer in the spent medium and titred for reactivated virus by infectious virus assay.

2.2.5 **Effective concentration 50% (EC_{50}) assay**

Serial dilutions are made using ECT10 and 4'-s-EtdU or ACV, pre-warmed to 37°C. 5 ml of each dilution is added to a bijoux containing $1x10^6$ BHK cells, which have been pre-incubated with 200 pfu of MHV-68, at 37°C for 1 hour. The contents of the bijouxs were then emptied into 60mm petri-dishes and incubated at 37°C for 4 days. The medium was then removed and the monolayers fixed with formyl-saline and stained with trypan blue. The plaque counts were established by microscopic examination of the stained monolayer. The EC_{50} value defined the anti-viral concentration that inhibited 50% of plaque formation.

2.2.6 **Establishment of cell lines persistently infected with MHV-68**

One million NS0 cells were infected with MHV-68 at a MOI of 5, in 2ml of LCLC medium. The inoculated cells were incubated at 37°C for 1 hour on a shaker and then transferred to a T75 flask containing 20ml LCLC medium. The cells were cultured at 37°C for 3 passages and then frozen in liquid nitrogen. All experiments were carried out on infected NS0 cells retrieved from $N_2(l)$.

$2x10^7$ MGC7 cells were infected with MHV-68 at a MOI of 1, in 5ml of the MHC7-growth medium. The inoculated cells were incubated at 37°C for 1 hour on a shaker and then transferred to 4 T175 flasks containing 50ml growth medium supplemented with 4'-s-EtdU, at either 2μg/ml or 0.2μg/ml (final concentration). The cell lines were then cultured as per normal using 4'-s-EtdU supplemented medium.

BHK cells were inoculated with serial dilutions of MHV-68 resulting in a MOI ranging from 4 to $4x10^{-6}$ pfu per cell. After a one hour incubation at 37°C on a shaker, $1.25x10^6$ cells, from each MOI were cultured at 37°C, in T25 flasks with 7ml of ECT10 supplemented with 4'-s-EtdU (2μg/ml final concentration). On reaching confluence the cells were passaged and transferred to T75 flasks containing 20ml 4'-s-EtdU supplemented medium. The cells were then further passaged, on reaching

confluence, and transferred to T175 flasks containing 50ml of 4'-s-EtdU supplemented medium. The cells were then maintained in T175 flasks.

2.2.7 **Cloning infected NS0 cultures**

Persistently infected NS0 cultures were diluted using NS0 cloning medium, to 1.5 cells/ml. 200µl was then pipetted in each well of 5 round bottomed 96-well plates (Nunc). When the cloning medium became exhausted 20 well per original culture were selected to be transferred to 24 well plates (Nunc) containing 1ml NS0 cloning medium per well. Each clonal NS0 culture was further expanded into 1 well of a 6-well plate (Nunc) containing 6ml of normal LCLC medium. The clones were then expanded into T25 flasks and frozen in liquid nitrogen.

2.2.8 **Mice used for *in vivo* experiments**

C57BL/6 females 3-4 weeks of age on arrival (Bantin and Kingman, Grimston, Aldbrough, Hull, UK).

BALB/c females 3-4 weeks of age on arrival (Bantin and Kingman, Grimston, Aldbrough, Hull, UK).

SCID females (on a BALB/c background) 4-5 weeks of age on arrival (Olac).

2.2.9 **Experimental infection of mice**

Both the intra-nasal and the intra-peritoneal routes of infection were used to experimentally introduce MHV-68 into mice. Mice were lightly anaesthetised with halothane (Rhone Merieux Ltd, Harlow, Essex) and inoculated (in) with $4x10^5$ pfu MHV-68, in 40µl, using a Gilson automatic pipette. Viral inoculum was derived from the viral stock, diluted to the right concentration with PBS (as previously described by Sunil-Chandra *et al*, 1992a). Alternatively, mice were injected intra-

peritoneally (ip), with $4x10^5$ pfu MHV-68, in 0.2 ml, using a 1ml sterile syringe (Becton and Dickinson) and a 27G hypodermic needle (Sherwood Medical). Viral inoculum was derived from the viral stock, diluted to the right concentration with PBS.

2.3.1 **Anti-viral administration**

For short term administration, lightly anaesthetised mice were orally injected with 0.2ml of a 0.5% (W/V) 4'-s-EtdU suspention (was made up with sterile distilled water) using a gavarge needle. For long-term administration, sterile 4'-s-EtdU stock solution was diluted with tap water (1/6) to give a final concentration of 0.33mg/ml. The 4'-s-EtdU solution was then filter sterilised and administered as drinking water for the long-term treatment of mice.

2.3.2 **Immune suppression of mice**

Cyclosporin A powder (1g) was mixed with 50ml ultra-pure olive oil (Sigma) and heating to 60°C and devolved under constant agitation, via a magnetic stirrer. The resultant 20mg/ml stock was stored at room temperature (RT). The CsA stock was injected into mice (ip) at 0.1ml per mouse twice weekly.

2.3.3 **Adoptive transfer of splenocytes**

Splenocytes were adoptively transferred into mice following 2 different protocols. For the pilot adoptive transfer of splenocytes, from infected mice, into SCID mice, spleens were removed from BALB/c donors 12 days post intra-peritoneal inoculation with $4x10^5$ pfu MHV-68. The donor mice had been place on 4'-s-EtdU drinking from day 6 post infection so as to prevent there being any infectious virions being present at the time of adoptive transfer. Lymphocytes were purified on a ficoll gradient and pooled. Half the splenocytes were mixed with YTS169 derived anti-CD8 monoclonal (1mg/$7x10^7$ cells). $7x10^7$ cells were then injected (ip) into the four

groups of SCID mice. Groups1 and 3 got whole lymphocytes and groups 2 and 4 got lymphocytes mixed with anti-CD8 antibody. Groups 3 and 4 were put on 4'-s-EtdU drinking water from 2 days prior to adoptive transfer of splenocytes. Two days after adoptive transfer groups 2 and 4 were injected (ip) with 1mg/mouse, in 0.1ml, of the YTS169 derived anti-CD8 monoclonal antibody.

For the main adoptive transfer of splenocytes into SCID mice, spleens were removed from uninfected BALB/c and infected BALB/c donors 12 days post intra-peritoneal inoculation with $4x10^5$ pfu MHV-68. The resultant whole splenocyte preparation were injected (ip) into the different groups of SCID mice, $4x10^7$ white cells per mouse. Groups 1 and 2 received uninfected splenocytes and groups 3 and 4 received uninfected splenocytes mixed with anti-CD8 antibody. Groups 5 received splenocytes from infected donors and groups 6 and 7 received splenocytes from infected donors mixed with anti-CD 8 antibody. Group 10 consisted of normal BALB/c mice, which received splenocytes from the infected donors.. The day after adoptive transfer groups 2, 3, 4, and 7 were injected ip with $4x10^4$ pfu MHV-68, as were groups 8 and 9 SCID mice, which did not receive an adoptive transfer. Two days after adoptive transfer groups 3, 4, 6 and 7 were given a second injection ip with anti-CD8 antibody, 1mg per mouse in 0.1ml. The mice from groups 4, 7 and 9 were put on 4'-s-EtdU drinking water from 5 days post adoptive transfer and until the experiment ended.

2.3.4 **Sampling of mice**

Mice were killed by cranial dislocation and the relevant organs removed by dissection. Organs, on removal, were placed in a bijoux containing 4ml of cold LCLC medium and placed on ice.

2.3.5 **Antibodies used**

Normal swine serum.

Normal sheep serum.

Long-term MHV-68 infected BALB/c mouse serum.

Rabbit (A) anti-MHV-68 hyper-immune serum.

YTS 191 rat anti-mouse CD4 ascites (NH4SO4 precipitated)

YTS 169 rat anti-mouse CD8 ascites (NH4SO4 precipitated)

Biotin conjugated goat anti-mouse IgM (Sera lab)

Biotin conjugated Sheep anti-mouse IgG heavy chain (Serotec)

Horseradish peroxidase (HRP) conjugated rabbit anti-mouse (DAKO)

Flouroscene (FITC) conjugated swine anti-rabbit IgG heavy chain (DAKO)

FITC conjugated Strepavidin (Serotec)

FITC conjugated anti-mouse CD45R (Pharminogen)

FITC conjugated star 49 goat anti-rabbit (Serotec)

Rat ascites were carried out, using LOUxDA F1 hybrids (as described by Cobbold *et al*, 1994)

2.3.6 **Collection of mouse sera**

Blood, including clots, was collected from the thorax of killed mice, using a sterile pasteur pipette, placed in a cryovial, and put on ice. The heart ventricles were snipped with dissection scissors of newly dead animals to maximise yield. Blood collection from live animals was achieved by tail bleeds. Mice were placed in a bucket positioned under a heating lamp for 2 minutes. The tail veins of the mice cut with a sterile scalpel. The blood drops were collected in a cryovial and put on ice. After approximately 10 minutes on ice, the clots in the blood samples were broken up by external agitation and then placed back on ice for a further 2 to 3 hours. The clots were then pelleted by centrifugation at 1800 RPM for 10 minutes in a Beckman TJ-6 centrifuge, set to 4°C. The serum was removed, placed in a sterile 1.5ml eppendorf tube and stored at -30°C (as previously described by Sunil-Chandra *et al*, 1992b).

2.3.7 **Cytosmear preparation and staining for MHV-68 antigens**

Reagents

Wash buffer - PBS with 0.2% (W/V) bovine serum albumin faction V (Sigma) and 0.02% (V/V) Tween 20 (Sigma).

Using a diamond tipped pen, six circles were drawn onto a bio-bonded slide. The circles were the traced around using a water repellent pen. Cell were suspended in 10% formyl-saline at a density of 10^7 cells per ml. 10µl were then pipetted onto a slide, using a 20µl Gilson, in the centre of a circle. The cells were spread though out the well using the tip of the Gilson and allowed to air dry in a fume hood. When dry the cells were rapped in aluminium foil and stored at -80°C.

Protocol

The cytosmears were removed from the -80°C and allowed to reach RT before the foil was removed. Before staining the slides were placed in rack and washed once with wash buffer. Each wash consisted of the rack, containing the slides, into a glass jar containing a magnetic stirrer. The jar was filled with wash buffer, stirred for 5 minutes at RT and then the wash buffer removed. The slides was then place face up across the two parallel plastic tubes within a damp box. The damp box comprised of a foil rapped plastic box containing 2 parallel plastic tubes of equal height attached to the bottom, surrounded by two pieces of damp cotton wool.

Two methods for staining for MHV-68 antigen expression was used. The first used rabbit anti-MHV-68 hyper immune sera as the primary detection antibody. To each cytosmear containing well, 50µl of normal swine blocking serum, (diluted 1/10) was added. With both methods the blocking serum used was the normal serum of the species from which the secondary antibody was derived and the different anti-bodies diluted with wash buffer. The lid was placed on the damp box and the cells were

incubated at 37°C for 30 minutes. The normal swine serum was removed and replaced with 50µl of rabbit anti–MHV-68 hyper immune serum (diluted 1/1000). The cells were then incubated, as before, for 30 minutes at 37°C. The primary antibody was then removed and the slides washed three times with wash buffer. The slide then had 50µl FITC-conjugated swine anti-rabbit serum (diluted 1/1000) added and the slides incubated as before. The secondary antibody was then removed and the slides washed a further three times.

The second of the 2 methods, used sera from long-term MHV-68 infected mice as the primary detection antibody. To each cytosmear containing well, 50µl of normal sheep blocking serum (diluted 1/10) was added. The lid was placed on the damp box and the cells were incubated at 37°C for 30 minutes. The normal sheep serum was removed and replaced with 50µl of sera from long-term infected mice, (diluted 1/50). The cells were then incubated, as before, for 30 minutes at 37°C. The primary antibody was then removed and the slides washed three times with wash buffer. The slide then had 50µl of biotin conjugated sheep anti-mouse (diluted 1/1000).added and the slides incubated as before. The secondary antibody was then removed and the slides washed a further three times and 50µl of streptavidin FITC (diluted 1/1000). The were then slides incubated as before, the streptavidin FITC removed and washed a further three times. The slides containing antibody bound cytosmears were mounted using aqueous mountant and a cover-slip. The cover-slip was sealed to the slide using nail-varnish and observed using a UV microscope.

2.3.8 **FACS analysis of splenocytes**

Reagents

1% formyl saline - 10% formyl-saline solution diluted 1/10 (V/V) with sterile PBS.

FACS buffer - Cytosmear wash buffer (see section 2.3.8) with 0.02% (W/V) sodium azide in PBS (all antibody working stocks were diluted in FACS buffer).

Procedure

FACS analysis of splenic lymphocytes was carried out (in accordance with Ehtisham *et al*, 1993) using splenocytes preparations, as previously described. $1x10^6$ cells were added to a FACS tube and the cells pelleted at 1400 RPM for 3 minutes in a Beckman TJ-6 centrifuge, set to 4°C. The cells were resuspended by external agitation, 50µl of primary antibody added and the tubes incubated for 20 minutes on ice. T-cells were stained with rat anti-mouse CD4 (1/1000) and rat anti-mouse CD8 (1/1000) monoclonal antibodies. B-cell were stained for using either FITC-conjugated anti-mouse CD45R (1/500) or biotin conjugated sheep anti-mouse IgM antibodies (1/200). The cells were then pelleted by centrifugation and the supernatants removed by aspiration. Each tube then underwent 3 washes with cold FACS buffer. Each wash consisted of adding 200µl of cold FACS buffer to a resuspended cell pellet and then repelleting the cells by centrifugation. 50µl of secondary antibody was then add and incubated on ice, for 20 minutes. The secondary antibodies for the anti–CD4 and anti-CD8 monoclonal antibodies was Star 49 (1/1000) and the secondary antibody for the anti-IgM was streptavidin labelled FITC (1/1000). The cells were then peletted by centrifugation, the supernatant removed and washed a further 3 times. The cells were finally fixed using 1% formyl-saline. The cells were counted using a FACStar (Becton Dickinson) FACS scanner.

2.3.9 **MHV-68 Enzyme-Linked Immunosorbent Assay (ELISA).**

Reagents

Borate buffered saline (BBS) - Boric acid (Sigma) 61.85g, disodium tetraborate (Sigma) 95.4g and NaCl (Sigma) 43.85g, made up to 10 litres with distilled water (pH 8.2).

BBS/Tween 20 - Tween 20 (Sigma) was added to BBS to make final concentration of 0.05% (V/V).

Carbonate/bicarbonate buffer (pH 9.8) - 22ml of sodium carbonate solution (0.2M) added to 28ml sodium bicarbonate solution (0.2M).

Citrate phosphate buffer (pH 5.0) - 0.1M citric acid (Sigma) solution was added to 0.2M disodium orthophosphate (Sigma) solution until pH reaches 5.0.

HRP substrate solution - 0.4ml O-phenylenediamine dihydrochloride (OPD) stock solution and 25µl of hydrogen peroxide solution (20 volumes) was added to 25ml citrate phosphate buffer (pH 5.0).

OPD stock solution - OPD 1g dissolved in 40ml of citrate phosphate buffer (pH 5.0). Store as frozen 0.4ml aliquots.

PBS/Tween 20 - Tween 20 (Sigma) was added to PBS to make final concentration of 0.05% (V/V).

Protocol

The mouse MHV-68 sera conversion ELISA was carried out (in accordance with Usherwood *et al*, 1996) on mouse sera diluted 1/20. To each well of an Immulon 4 plate (Dynatech), 100µl of Rabbit A anti-MHV-68 hyper-immune sera diluted 1/1000 in carbonate/bicarbonate buffer (pH 9.8), was added and incubated over night at 4°C. The wells were then blocked for 1 hour at 37°C with 100µl of 2% (V/V) normal rabbit serum, diluted in BBS/Tween 20. The plates were then washed 6 times with BBS/Tween 20, 100µl per well. MHV-68 stock ($3.8x10^8$ pfu/ml) was UV irradiated for 30 minutes. The irradiated MHV-68 was diluted 1/64 in PBS and then added to the plate (100µl per well) and incubated for 1 hour at 37°C. The plates were then washed 6 times with BBS/Tween 20 (100µl per well). 200µl of the test and

control sera, diluted in 1% normal rabbit sera in PBS/Tween 20, was added to each well and incubated over night at 37°C. The test mouse sera samples were diluted 1/20. Hyper-immune mouse sera, diluted 1/400, was used as a positive control and pre-bleed mouse sera, diluted 1/20, were used as negative controls. The plates were then washed 6 times with BBS/Tween 20 (100µl per well). 100µl of HRP conjugated rabbit anti-mouse (diluted 1/2000 in 1% normal rabbit serum dissolved in BBS/Tween 20) was then added to each well and incubated for 1 hour at 37°C. . The plates were then washed 6 times with BBS/Tween 20 (100µl per well). 100µl of HRP substrate solution was then added to each well and incubated at room temperature. After 15 minutes the reaction was stopped with 100µl of 12.5% (V/V) sulphuric acid. The sample OD value was taken at 490nm using an ELISA plate reader (DYNATECH MR5000).

2.4.1 **DNA extraction and purification**

QIAamp tissue kits (Qiagen) reagents (50 preparations)

AL buffer - Add the contents of reagent AL1 (Qiagen) to the reagent AL2 (Qiagen) solution and mix thoroughly by shaking. The buffer was store at RT in the dark.

ATL buffer (Qiagen) - Stored at RT.

AW solution - 40ml of 99.7-100% Ethanol (BDH) was added to the AW stock bottle, mixed by shaking and then stored at RT.

Proteinase K stock - 1.4ml of distilled water was added to the lyophilised proteinase K (Qiagen) powder and stored at -20°C in 100µl aliquots.

Protocol

DNA extraction from both tissues and *in vitro* cell lines was achieved using QIAamp

tissue kits (Qiagen) in accordance with the manufactures instructions. Approximately $5x10^6$ cells, 25mg of tissue or 80µl of tissue homogenate was placed in a 1.5ml microfuge tube and made up to 180µl with ATL buffer. 20µl of proteinase K stock solution was added to each tube and incubated at 55°C, vortexing frequently, until sample was completely digested. 200µl of AL buffer was then added, the tubes vortexed and then incubated for 10 minutes at 70°C. 210µl of 99.7-100% ethanol (BDH) was then added and the tubes and vortexed. A QIAamp spin columns were placed in 2ml collection tube and the cell / tissue lysates pipetted into the columns. The columns were centrifuged in a Micro centaur (MSE) microfuge at 7500 RPM for one minute. The columns were placed in fresh collection tubes and 500µl of AW buffer added to each column. The columns were re-centrifuged as before for one minute and place in a fresh collection tube. A further 500µl of AW buffer was added to each column and the columns were centrifuged as before for 3 minutes. 100µl of distilled water pre-heated to 70°C was added to each tube, incubated for 1 minute and then centrifuged as before for 1 minute into a 1.5ml eppendorf.

2.4.2 **DNA quantification**

Reagents (Hoefer Pharmacia Biotech Inc.)

Hoechst (H) 33258 stock - 10ml of SDW was added to 10mg H 33258 powder to make a 1mg/ml stock solution. The solution was stored in the dark at 4°C.

Tris normal saline EDTA (TNE) buffer (10X) stock solution - 100mM Tris (Sigma), 10mM EDTA and 2M NaCl made up in distilled water and adjusted to pH 7.4 using 10M Hydrochloric acid (HCl). The solution was then autoclaved and stored at RT.

Solution A - 10ml of TNE (10X) and 10µl of H33258 was added to 90 ml SDW and stored in the dark at 4°C.

Standardising DNA stock - 100µl calf thymus DNA (1mg/ml) standard was mixed with 100µl of TNE (10X) and 800µl of SDW. The resultant DNA solution (100µg/ml) was stored at 4°C.

Protocol

DNA samples were quantified using a DyNA Quant 200 Fluorometer (Hoefer Pharmacia Biotech Inc.), in accordance with the manufacturer's instructions. 2ml of Solution A and placed in a quartz cuvette and used to blank the fluorometer. 2µl of Standardising DNA was added to the cuvette, mixed thoroughly and re-read to calibrate. The unknown DNA samples were read in the same manner as with the calibration of the apparatus.

2.4.3 **MHV-68 detection by Polymerase Chain reaction**

Taq DNA polymerase kit (Boehringer Mannheim Ltd).

Taq stock - Recombinant Taq DNA polymerase (5units/µl) in 20mM Tris-HCl (pH 8.0), 0.1mM EDTA, 1mM DTT and 50%(V/V) glycerol.

PCR buffer (10X) - 200mM Tris-HCl (pH 8.4) and 500mM KCl.

Magnesium solution - 50mM MgCl.

dNTP Set (Pharmacia Biotech) - 2'-Deoxyadenosine 5'-triphosphate (dATP) 100mM, 2'-Deoxycytidine 5'-triphosphate (dCTP) 100mM, 2'-Deoxyguanosine 5'-triphosphate (dGTP) 100mM, and 2'-Deoxythymidine 5'-triphosphate (dTTP) 100mM.

Primers - PAG 1 and 2 were diluted 1/30 and PAG 11 and 12 diluted with 1/5, both with distilled water and stored at -20°C (*see figure 2.1*).

Figure 2.1

748 CCTTGGTGCGGCCGAGACTGTAGAGGGTATTACAAGTAGAGAGATGGAAA

799 TAAATGCTACAAAGGCACCATCTAGCGGTGCAACATTTTCATTACTAGTA

848 ACTCTCTCGAACAACAATCCCACTACAATTATGCGCCCCCCTGTGGCCCA

899 AAATG**GTGAGAGTGTACACAAAGACG**CCCGCAGCGCTTCCGCGTCCGATC

949 CCAC**CACCTCAGAACCAACTTCC**CCAGGAGAGGAGCCAACGGAGGCTGAC

999 CCCAAAGCGGCACCGTCCGCTGGGCATGTTGGGGAGACTGAGCCCGAGTC

1048 TCCAACCCCTCTACCCGCAACACCTAAACCCTCCTCACAGGAAGATAATC

1099 CAACTATGACTCCCCCTACAGCAGAACCTCCCACCTCCAATGCAGATGTT

1148 TCTACTGAACACGTTGATGAGACAGAACCCGAGTCACCAACCTTCCTCCC

1199 CCCTACTCCCGAACCAGACACCCCCACAACCCCGGAAACAACCACCCCT

1248 TCCCAAAATCAGGAAGATGAACCCACCCTAACTACTTCTTCATCGGACGCT

1299 C**CTGCTGACACATCAGATAC**CAGTCCTCCCAAACAAGAGGATGACCCAGT

1348 AAAACCTACAGAGTCCAAACCCCAG**GCTGAACCAAAGGA**CAATTCTCCTT

1399 CCGATGTACCTGAAACCGCTGACAGTCCGACAGATCCCGCCTCCCCTACC

1448 GTCGAACTCACCCCTCCCACAGAGCCCCCTACCCCCGAAACCGTGTCTCC

1499 AGCCGATTCCCCAGTTCCTCAACCAACTGCCCCAGCTGAACCTTCAAAGC

1548 CAGAACCTACACCACCCGTAGACCCGCCGGCTACTGAACCAAATACCCCA

Figure 2.1 The nucleotide sequence of ORF M7 (BPRF1) that is amplified by the PCR primers PAG 1 and 2 (red) and PAG 11 and 12 (blue). The arrows denote the direction of extension from the primers. The sequence is numbered from the upstream BamHI site which is the boundary between the BamHI P_2 and N fragments according to the map of Efstathiou et al, 1990b. The figure was adapted from Stuart et al, 1996.

PAG1 - 5' GTA GGA TCC GTG AGA GTG TAC ACA AAG ACG C 3' (sense)

PAG2 - 5' GGA GAA TTC TCC TTT GGT TCA GC 3' (anti-sense)

PAG11 - 5' CAC CTC AGA ACC AAC TTC 3' (sense)

PAG12 - 5' GTA TCT GAT GTG TCA GCA G 3' (anti-sense)

2.4.4 **First round PCR**

The first round of PCR was carried out as previously described (Stewart *et al*, 1996). The PCR cocktail mix was made up with 77µl Ultra pure de-ionised water (Sigma), 10µl PCR buffer (Boehringer Mannheim), 3µl magnesium solution (Boehringer Mannheim), 1µl dNTP stock (Pharmacia Biotech) and 1µl of both PAG1 and PAG2 (the volumes were multiplied up depending on the number of tubes taking part). 93µl was then added to each tube and 5µl of sample DNA (100ng/µl l) was added and the samples put in the PCR machine (Hybaid OmniGene - with heated lid). The Taq was added after a hot start. For the first cycle, the blocks were set, using tube control, at 45°C for 1 minute to bring the tube contents up to temperature, then 95°C for 5 minutes, to melt the DNA, and finally 1 minute at 56°C to allow primer annealing. The temperature was held at 56°C while the Taq was added, 2µl diluted 1/5 with 1X reaction buffer. The second cycle consisted of a 72°C elongation step, for 1 minute, a 94°C re-melting step, for 1 minute and a 56°C re-annealing step for one minute. This was repeated 40 times and then finally there was a 5 minute final extension at 72°C.

2.4.5 **Second round (nested) PCR**

The second round of PCR was carried out as previously described (Usherwood *et al*, 1996). The PCR cocktail mix for the second round of PCR was made up the same as the first but 1µl of PAG11 and PAG12 was added instead of PAG1 and PAG2. 5µl of first round PCR product was added to 93µl of the second round cocktail mix in a fresh 750µl eppendorf and placed in the PCR machine. For the first cycle, the blocks

were set, using tube control, at 45°C for 1 minute to bring the tube contents up to temperature, then 95°C for 5 minutes, to melt the DNA, and finally 1 minute at 49°C to allow primer annealing. The temperature was held at 49°C while the Taq was added, 2µl diluted 1/5 with 1X reaction buffer. The second cycle consisted of a 72°C elongation step, for 1 minute, a 94°C re-melting step, for 1 minute and a 49°C re-annealing step for one minute. This was repeated 25 times and then finally there was a 5 minute final extension at 72°C.

2.4.6 **Visualising PCR products**

Reagents

Agarose (Flowgen) - SeaKem LE agarose.

Ethidium bromide stock (Sigma)- 5mg/ml. Stored in the dark at 4°C.

Tris acetate EDTA (TAE) buffer (50X) - 24.2% (W/V) Tris base (Sigma), 5.71% (V/V) concentrated acetic acid (Sigma) and 10% (V/V) 0.5M EDTA (pH 8.0).

Loading buffer (10X) - 15% (W/V) Ficoll type 400 and 0.25% bromophenol blue in SDW.

1Kb ladder (GibCo BRL) - 200 to 1200 mg/ml in 10mM Tris-HCl (pH 7.5), 50mM NaCL and 0.1mM EDTA.

Protocol

PCR products were visualised by agarose gel electrophoresis. Gels were made by adding 2% (W/V) agarose powder to distilled water containing 2% (V/V) TAE(50X). The mixture was boiled in a microwave and swirled until the agarose was completely melted and had gone into solution. The solution was then cooled in a 60°C water

bath. The agarose solution was then poured in the assembled gel apparatus (GibCo BRL Horizon™ and allowed to solidify for approximately 2 hours. TAE Buffer (1X) was made by diluting the TAE stock solution 1/50 with distilled water and pour into the gel apparatus so the gel was immersed. The PCR samples were mixed with loading buffer (9:1 respectively) and loaded onto the gel. The gels were run at 60 volts (rate limiting step), using a power pack (Pharmacia LKB) for approximately 3 hours. The gels were then placed in a plastic container containing approximately 300ml of ethidium bromide solution (250ng/ml diluted in distilled water) and incubated for 20 minutes on a shaker (set to low speed). The ethidium bromide was then removed and replaced with 1 litre of distilled water and allowed to de-stain for 20 minutes on the shaker. The DNA was visualised using a UV transilluminator (UVP Inc.) and an image analyser (Mitsubishi).

Chapter 3: The inhibitory properties of 2'-deoxy-5-ethyl-β-4'-thiouridine on MHV-68 infection of *in vitro* cell lines.

3.1.1 Summary

The inhibitory properties of 2'-deoxy-5-ethyl-β-4'-thiouridine (4'-s-EtdU) on the replication of MHV-68 was studied in a number of different *in vitro* cell lines. The potency of 4'-s-EtdU was determined to be 35 ng/ml, by EC_{50} assay and was shown to be over 10 fold more potent than ACV. 4'-s-EtdU treatment of persistently MHV-68 infected NS0 cells, effectively eliminated productive virus replication but failed to significantly effect the number of latent virus infected cells in the culture. At 2μg/ml, 4'-s-EtdU long-term treatment of persistently infected NS0 cells suppressed the reactivation of latent virus for up to 3 weeks after withdrawal. However, 4'-s-EtdU treatment at 0.2μg/ml failed to have any long-term effects on viral reactivation after withdrawal of 4'-s-EtdU. Interestingly, MHV-68 could not be eliminated from cell lines which ordinarily support only lytic virus replication. 4'-s-EtdU treatment protected MGC7 cells, a transformed glial cell line, from the cytolytic effects of infection with MHV-68 and eliminated viral antigen expression. However, treatment could not eliminate MHV-68 from the cells. BHK cells were less readily protected from the cytotoxic effects of MHV-68 infection by 4'-s-EtdU. Cultures infected at a MOI of 4 or 0.4 pfu per cell were either not protected or only partially protected. 4'-s-EtdU however did protect cultures infected at a MOI of 0.04 pfu per cell or less. Long-term treatment of MGC7 cells and BHK cells lead to the spontaneous generation of 4'-s-EtdU-resistant viruses. However, infected BHK cells gave rise to 4'-s-EtdU resistant virus variants over shorter periods of time than the MGC-7 cells. Six 4'-s-EtdU resistant virus isolates were cloned from persistently infected MGC-7 cells and the 4'-s-EtdU resistance was found to be independent of ACV resistance. No reduction in ACV sensitivity was observed in any of the six cloned 4'-s-EtdU resistant isolates and therefore resistance was unlikely to be due to the functional deletion of the virus encoded TK gene.

3.1.2 Anti-herpesvirus agents

There are a great number of compounds and reagents that have anti-viral properties, both *in vitro* and in *vivo*. They act either on the target cells, (i.e. interferon) or directly on the virus, by disrupting replication at different stages of the infectious cycle. Anti-viral reagents have been generated to disrupt cellular attachment (i.e. antibodies), fusion/uncoating (i.e. antibodies and amantadine), viral DNA and RNA polymerase activity (i.e. pyrophosphate analogues and non-nucleoside reverse transcriptase inhibitors) and post translational modification (i.e. viral protease and glycosylation inhibitors). However, the most effective inhibitors of herpesvirus replication are the nucleoside analogues (for general reviews, see Hirsch *et al*, 1990 and Bean 1992). Nucleoside analogues are generally good at combating lytic viral infections but appear ineffective at controlling latent virus infections, either *in vivo* (Field *et al*, 1979, Yao *et al*, 1989 & Sunil-Chandra *et al*, 1994c) or *in vitro* (Lin *et al*, 1985, Lin *et al*, 1991 & Sunil-Chandra *et al*, 1993). As with all anti-viral therapies, there are a number of disadvantages with nucleoside analogue treatments. The two main disadvantages are toxicity and restricted virus range. All nucleoside analogues, with perhaps the exception of ACV, tend to be toxic and therefore have narrow therapeutic windows. They also tend to be effective at inhibiting only a narrow range of different viruses. Nucleoside analogues that are effective on a large range of viruses tend to have high toxicity. Other problems associated with nucleoside analogues arise through their mode of action. Nucleosides once phosphorylated become 'activated'. In this active form, they become recognised by viral nucleic acid polymerases, in preference to cellular DNA polymerases, and get incorporated into replicating viral genomes. This interferes with replication, generally during elongation, either by inducing chain termination or by less well defined mechanisms. Because cellular DNA polymerases can also incorporate activated nucleoside analogues and because there is always, with the exception of ACV, a certain amount of "read through", this can induce mutations which have

potential carcinogenicity or embryo toxicity in the patients.

3.1.3 **Virus activated anti-virals**

Nucleoside analogues can be split into 2 groups, those that are preferentially phosphorylated by viral kinases and those that are phosphorylated by cellular kinases. However, all anti-viral nucleoside analogues are believed to work in a similar manner. The nucleoside analogue, once phosphorylated at the 5' position, mimics one of the 4 naturally occurring dNTPs and is incorporated into replicating viral genomic DNA (or RNA). The effect of incorporation brings about a premature termination of DNA (or RNA) polymerisation. This occurs either by direct termination of elongation or by inducing structural aberrations in the expanding duplex and so destabilising the viral polymerase complex.

Examples of nucleoside analogues which appear exclusively phosphorylated by viral TK are ACV (9-[2 hydroxy ethoxymethyl guanine]), 9-(4-hydroxyl-3-hydroxy methylbut-1-Y) Guanine, termed penciclovir (PCV), (E)-5-(2-bromovinyl)-2'-deoxy-uridine (BVdU), and 1-β-D-arabinofuranosyl-(E)-5-(2-bromovinyl) uracil (BVaraU). As previously described, viral TK is encoded by a partially conserved gene found throughout the alpha and gammaherpesviruses (*see Table 1.3*). ACV is an acyclic guanosine analogue, which contains a positional equivalent of a 5' hyroxyl group but not a positional equivalent to the 3' carbon. ACV will not therefore act as a substrate for 5' phosphorylation, by cellular TK. However, due to the promiscuous nature of the enzymes encoded by herpesviruses for nucleotide metabolism, ACV will act as a substrate for the TK homologue encoded by many herpesviruses. ACV is phosphorylated efficiently by alphaherpesviruses, such as HSV-1, HSV-2 and VZV but less efficiently by gammaherpesviruses, such as EBV and HVS (Boon *et al*, 1997). ACV will inhibit the productive replication of EBV but not HVS. Betaherpesviruses, such as HCMV, do not code for a TK homologue and so fail to significantly activate ACV above background levels.

PCV is chemically similar to ACV, though unlike ACV, PCV does have a positional equivalent of a 3' hydroxyl group. The 3' hydroxyl group allows 'read through' to occur after incorporation into elongating DNA and so making PCV a non-obligate chain terminator. Despite this, the anti-viral activity associated with PCV appears due to incomplete synthesis of DNA during elongation. *In vitro* PCV inhibits HSV I, HSV II, VZV and EBV at similar concentrations as ACV (Ertl *et al*, 1995, Bacon 1995, 1996a & b). PCV is activated by HSV I TK more efficiently than AVC but HSV I polymerase has a higher affinity for ACV triphosphate (TP) than PCV-TP (Bacon *et al*, 1996b). PCV has intracellular half life of 10, 20 and 9 to 14 hours in HSV I, HSDV II and VZV infected cells respectively. This compares to 0.7, 1 and 0.8 hours respectively for ACV, giving PCV the far superior intracellular half life (Crumpacker *et al*, 1996 and Bacon *et al*, 1996b & c). Unlike ACV and PCV, BVdU and BVaraU are both pyrimidine analogues and are both highly potent at inhibiting VZV.

Other nucleotide analogues that are preferentially activated by viral kinases are 9-[1,3-dihydroxy-2-propoxyl] methylguanine, known as ganciclovir (GCV), 5'-iodo-2'-deoxyuridine (IDU) and triflurothymidine (TFT). GCV can be activated by both TK and a second herpesvirus specific kinase, encoded by homologues of the HCMV UL97 gene. The HCMV UL97 gene is a core herpesvirus gene (*see Table 1.2*) making GCV a highly potent anti-viral, with a broad herpesvirus range. As well as inhibiting the replication of HCMV, GCV is also able to inhibit HSV-1, HSV-2, VZV and EBV. However, because GCV along with IDU and TFT can be in part activated by cellular enzymes they have a greater toxicity than either ACV or PCV (Thust *et al*, 1996 and Lalezari *et al*, 1996).

3.1.4 **Non virus activated anti-virals**

The second group of nucleoside analogues, rely on cellular kinases for activation. Their specific anti-viral properties are due to the viral DNA (or RNA) polymerase having a higher substrate affinity for the activated nucleoside analogue, than cellular

DNA polymerases. In this way the anti-viral dose is set to be too low to be recognised by host cell polymerase, but high enough to inhibit viral replication. In general these nucleoside analogues will inhibit the replication of a broad range of different viruses, including both DNA and RNA viruses, but are highly toxic. 9-b-D-arabinofuranosyl adenine, known as vidarabine (ara A), has broad anti-herpesvirus specificity, though shows reduced effects against EBV and CMV. As with other analogues in this group, such as 1-D-ribofuranosyl-1,2,4-triazole-3-carboxamide, known as ribavirin, and 3[-azido-3'-deoxythymidine], known as zidovudine (AZT), ara A has anti-viral activity against a number of RNA viruses. Ribavirin is used to treat lassar fever and AZT to treat HIV infection. The requirement for virus activation of an anti-viral can be a disadvantage. The anti-viral nucleotide analogue cidofovir (HPMPC or GS 504) is an acyclic analogue of cytodine-monophosphate. Cellular kinases convert HPMPC to the triphosphate form and so anti-viral activity does not require viral TK. HPMPC has activity against alpha, beta and gammaherpesviruses, including TK negative isolates, as well as papillomaviruses. The cellular half life for HPHPC is also greatly in excess of normal nucleoside analogues (Alrabiah *et al*, 1996 and Freeman *et al*, 1996).

3.1.5 **2'-deoxy-5-ethyl-β-4'-thiouridine**

2'-deoxy-5-ethyl-β-4'-thiouridine (4'-s-EtdU) is a new nucleoside analogue, developed by GlaxoWellcome, as one of a large batch of different 5'-substituted 2'-deoxy-4'-thiouridine analogues. Unlike ACV, PCV or GCV, which are acyclic purine analogues, 4'-s-EtdU, as with BVdU and BVaraU, is a cyclic pyrimidine analogue. Along with the addition of an ethyl group in the purine ring, 4'-s-EtdU also has a sulphur atom substituted for the 4' oxygen in the deoxyribose chain (*see figure 3.1.1*). Substitution of the 4' oxygen atom reduces the susceptibility of 2'-deoxynucleosides to degradation by nucleoside phophorylases. Although 4'-thioribonucleosides have been shown to have anticancer properties, little is known about their potential anti-viral effects (Secrist *et al*, 1991). A study into 4'- oxygen substituted, thio-analogues has shown that the thio-analogue of BVdU (4'-s-BVdU)

Figure 3.1.1

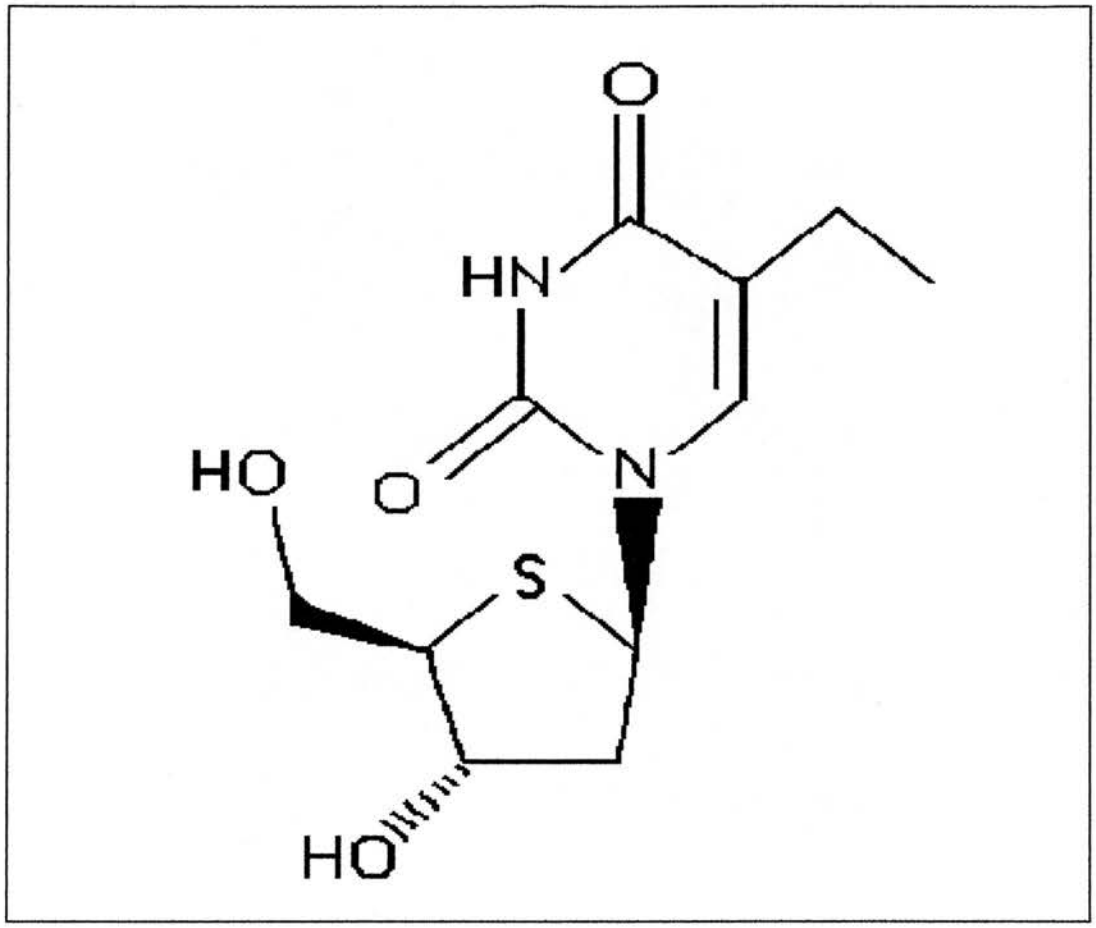


Figure 3.1.1 The structural formula of 2'-deoxy-5-ethyl-β-4'-thiouridine (4's-EtdU). 4'-s-EtdU is a cyclic pyrimidine nucleoside analogue with a sulphur atom substituted for the 4' oxygen atom in the deoxyribose chain. Activation (5' phosphorylation) is required for incorporation into elongating DNA, however the exact mechanism of inhibition of viral replication is unclear, since 4'-s-EtdU is not an obligatory chain terminator.

remains highly active against HSV-1, HSV-2 and VZV. However, the 4'-thio analogue of AZT (4'-s-AZT) is rendered inactive against HIV infection (Eltorgman *et al*, 1992, Rahim *et al*, 1996 and Basnak *et al*, 1998).

4'-s-EtdU is specifically activated by herpesvirus TK, and appears to have a low toxicity, both *in vivo* and *in vitro*. 4'-s-EtdU is activated, via phosphorylation of the 5' hydroxyl group and it has a greater combined over all potency against alphaherpesviruses, such as HSV-1, HSV-2 and VZV, than either BVdU, ACV or PCV (Rahim *et al*, 1996). 4'-s-EtdU appears to be active against a similar range of viruses as is ACV and hence does not inhibit the replication of HCMV (*see Table 3.1*). Prior to this study the potency of 4'-s-EtdU against gammaherpesviruses, such as MHV-68, had not been investigated. However a preliminary study had shown that 4'-s-EtdU is a potent inhibitor of MHV-68 productive replication both *in vitro* and *in vivo*, and has a low toxicity in mice (Barnes *et al*, unpublished data). The precise mechanism by which 4'-s-EtdU inhibits the productive replication of susceptible herpesviruses is at present unclear, since in activated form, unlike ACV, it is not an obligate chain terminator (GlaxoWellcome personal communication).

3.1.6 **The effect of ACV on the productive replication of MHV-68 *in vitro***

MHV-68 will readily form plaques, due to lytic replication, in fibroblastoid and epithelial *in vitro* cell lines. It is therefore possible to determine, by plaque reduction (EC_{50}) assays, the concentration of an anti-viral necessary to inhibit 50% of a fixed number of viral pfu. Previous studies testing the potency of ACV at inhibiting the lytic replication of MHV-68 have reported the EC_{50} of ACV to be 200ng/ml (Sunil-Chandra *et al*, 1994). Since MHV-68 is sensitive to ACV it was considered likely that MHV-68 would also be sensitive to 4'-s-EtdU.

3.1.7 **The effect of ACV on the latent replication of MHV-68 *in vitro***

EBV does not readily form plaques in *in vitro* cell lines and hence it is not possible

Table 3.1* **The comparative inhibitory effect of 4'-s-EtdU as compared to 3 leading anti-herpesvirus drugs.**

Compound	EC_{50}[a] (μM)			
	HSV-1	HSV-2	VZV	HCMV
4'-s-EtdU	0.17-0.5[b]	2-5[b]	0.99	138
ACV[cf]	1.8	1.7	46.8	85
PCV[df]	3.9	12.8	78.7	>100
BVdU[e]	1.4	10	<1	>100

** Table adapted from Rahim et al, 1996.*

Key: *[a] The micromolar concentration of the drug required to inhibit viral plaque formation by 50% over a 4 day period. The EC_{50} assay for HSV-1 and HSV-2 were carried out using Vero cells lines and for VZV and HCMV, MRC-5 cell lines. [b] Concentration depending on the specific strain used. [c] Acyclovir. [d] Penciclovir. [e] (E)-5-(2-bromovinyl)-2'-deoxy-uridine. [f] The mean EC_{50} concentration for a variety of strains.*

to determine the potency of an anti-viral by performing EC_{50} assays. An alternative to the EC_{50} assay is to super-infect RAJI cells with EBV in the presence of a test anti-viral. RAJI cells are an EBV positive, BL derived, *in vitro* cell line. Super-infection of RAJI cells induces an abortive productive infection, elevating the viral copy number from approximately 50 to between 300-600 viral genome copies per cell. In the presence of an effective EBV replication inhibitor, the copy number decreases to approximately 30 copies per cell. The rate at which this occurs and the converse after withdrawal of treatment, denotes the potency of the anti-viral. This method has been used to compare different anti-herpesvirus nucleoside analogues, including BvdU and ACV (Lin *et al*, 1984, 1985 & 1991 and Vanderhorst *et al*, 1987).

A similar, if not directly parallel, *in vitro* model has been established with MHV-68, in the form of MHV-68 infected B-cell myoloma derived, *in vitro* cell lines, such as NS0 cells. MHV-68 will persistently infect NS0 cells, giving rise to a chronically infected population, that persistently shed virus, as well as appearing to establish latency. Persistently infected lines contain both circular as well as linear viral genomic DNA, as determined by Gardella gel. After 2 weeks of culturing in the presence of 25 μg/ml of ACV, the linear genome cell content was greatly reduced, but the circular content was not. ACV was shown to eliminate productive virus from the cultures, but not latent, measured by infectious virus and co-cultivation assay, respectively (Sunil-Chandra *et al*, 1993). Persistently infected NS0 cultures provides a useful model for the study of the effects of an anti-viral on the dynamic state between viral latency and productive replication, over variable periods of time. The model also provides a system by which the relative concentration of an anti-viral, necessary for the complete elimination of productive viral replication in a culture, can be determined. This concentration tends to be greater than the minimal concentration required to prevent plaque formation, the EC_{100} value, since limited viral productive replication can potentially occur in the absence of plaque formation.

3.1.8 **Mixed glial clone 7 (MGC7)**

Cell lines other than epithelial and fibroblastoid cell lines are also susceptible to lytic infection by MHV-68. One such line is the *in vitro* transformed monoclonal glial cell line MGC7. MGC7 were derived from mixed glial cell cultures derived from the brains of 2 day old neonatal CBA mice. The glial cells were then infected with the tsa 58.3 recombinant retrovirus (Jat *et al*, 1989), allowing the stable expression of a temperature sensitive mutant of the SV40 large T antigen, in the infected cells. At the permissive temperature, 33°C to 34°C, the large T antigen expression transformed the cells allowing for the cloning out of 8 different lines. MGC7 are a monoclonal cell line of astrocytic lineage, expressing the astrocyte markers A2B5 and GFAP, but not oligodendrocyte markers, such as glactocerebroside, MOG and CNPase. The cells are a rapidly dividing, adherent cell line when cultured at 34°C but fail to grow when cultured at the non-permissive temperature, 40°C (Terry *et al*, 1997).

3.1.9 The inhibition of MHV-68 lytic replication in BHK cells

To determine the relative concentration of 4'-s-EtdU required to inhibit the lytic replication of MHV-68 *in vitro*, 4'-s-EtdU and ACV EC_{50} assays were carried out, in parallel, on MHV-68 working stocks. The EC_{50} for ACV was found to be 450 ng/ml (2μM) and for 4'-s-EtdU 35ng/ml (0.13μM) (*see figure 3.1.2*). From this result not only does 4'-s-EtdU inhibit the productive replication of MHV-68 but does so at a 15 fold lower molar concentration than ACV. The minimum concentration of 4'-s-EtdU required to prevent 100% plaque formation over a 4 day period was 200 ng/ml (0.73μM).

3.2.1 The establishment of MHV-68 persistently infected NS0 cells

To further characterise the anti-viral effects of 4'-s-EtdU, a persistently infected NS0 cell line was established. NS0 cells were infected at a MOI of 4 and cultured for 3 passages, to dilute out any input infectious virus, before being frozen in liquid nitrogen. NS0 cells were then retrieved from the $N_2(l)$ store and cultured for a further 3 passages prior to being treated with 4'-s-EtdU. The culture was split into 3 sub-cultures. The first remained untreated, the second and third were cultured in medium containing 0.2 μg/ml and 2 μg/ml 4'-s-EtdU respectively. These concentrations were chosen because 0.2 μg/ml 4'-s-EtdU represented the IC_{100} concentration, where as 2 μg/ml 4'-s-EtdU represented a biologically saturating dose. The persistently infected NS0 cells were cultured for 6 weeks with their respective doses of 4'-s-EtdU. After 3 weeks of culture the 4'-s-EtdU treatment was withdrawn from a flask of each of the treated cultures and then cultured for a further 3 weeks in the absence of 4'-s-EtdU. The cultures were passaged every 3 to 4 days and cells kept for infectious centre and infectious virus assays.

Figure 3.1.2

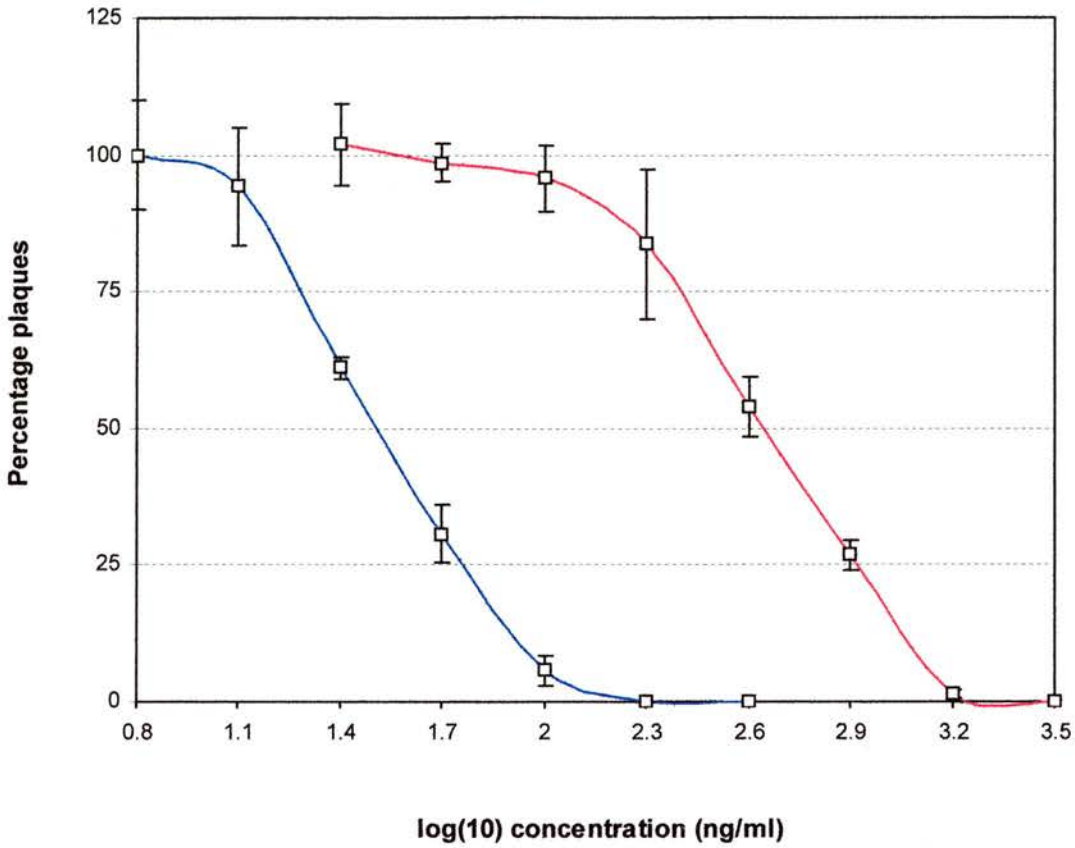


Figure 3.1.2 A typical plaque reduction profile for MHV-68 against ACV (red) and 4'-s-EtdU (blue), as determined by EC_{50} assay. The assay was performed in parallel, for both ACV and 4'-s-EtdU. The dotted lines represent the 100%, 75%, 50% and 25% plaquing values, as determined by the mean number of plaques observed in the absence of either ACV or 4'-s-EtdU.

3.2.2 **The inhibitory effects of 4'-s-EtdU on the productive replication of MHV-68 in persistently infected NS0 cells**

Treatment of infected cultures with 0.2 μg/ml 4'-s-EtdU did not completely eliminate cell associated infectious virus, although a 1000 fold decrease in titre was observed (*see figure 3.2.1A*). The amount of cell associated infectious virus produced by the cultures steadily declined for the first 3 weeks of treatment then levelled off at approximately 20 pfu per $2.5x10^6$ cells, from 24 days post treatment. On withdrawal of the 4'-s-EtdU treatment, the cell associated infectious virus titres remained constant for the first week, then gradually increased, reaching the original pre-treatment level by 18 to 22 days post withdrawal.

Treatment of the infected cultures with 2 μg/ml 4'-s-EtdU completely eliminated productive virus replication, as determined by both infectious virus assay (*see figure 3.2.1B*) and by immunostaining for late viral antigens (*see figure 3.2.2*). After 7 days of treatment, the cell associated infectious virus titre was reduced to 25 pfu per $2.5x10^6$ cells. After 14 days of treatment the cell associated infectious virus titres became undetectable and remained undetectable for the duration of treatment. After withdrawal of the 4'-s-EtdU medium supplement, a low level of cell associated infectious virus was detected in the cultures. However, the titres remained low (160 +/- 48 pfu per $2.5x10^6$ cells) and did not return to the original pre-treatment level. This compares with the mean infectious virus titre for the untreated culture of $2.2x10^4$ (+/- $4.8x10^3$) pfu per $2.5x10^6$ cells, over the entire time course.

Despite being cultured with 4'-s-EtdU for over 3 months, no evidence for the spontaneous generation of 4'-s-EtdU resistant virus variants was found in the persistently infected cultures. EC_{50} assays were carried out on virus preparations, generated from cells that were continuously cultured with 4'-s-EtdU at both 0.2 and 2μg/ml, for 47 days. These were compared with both the virus preparation generated from untreated NS0 cells and the original virus working stock, used to establish the persistently infected cell lines (*see figure 3.2.3*). However, no obvious differences in

Figure 3.2.1

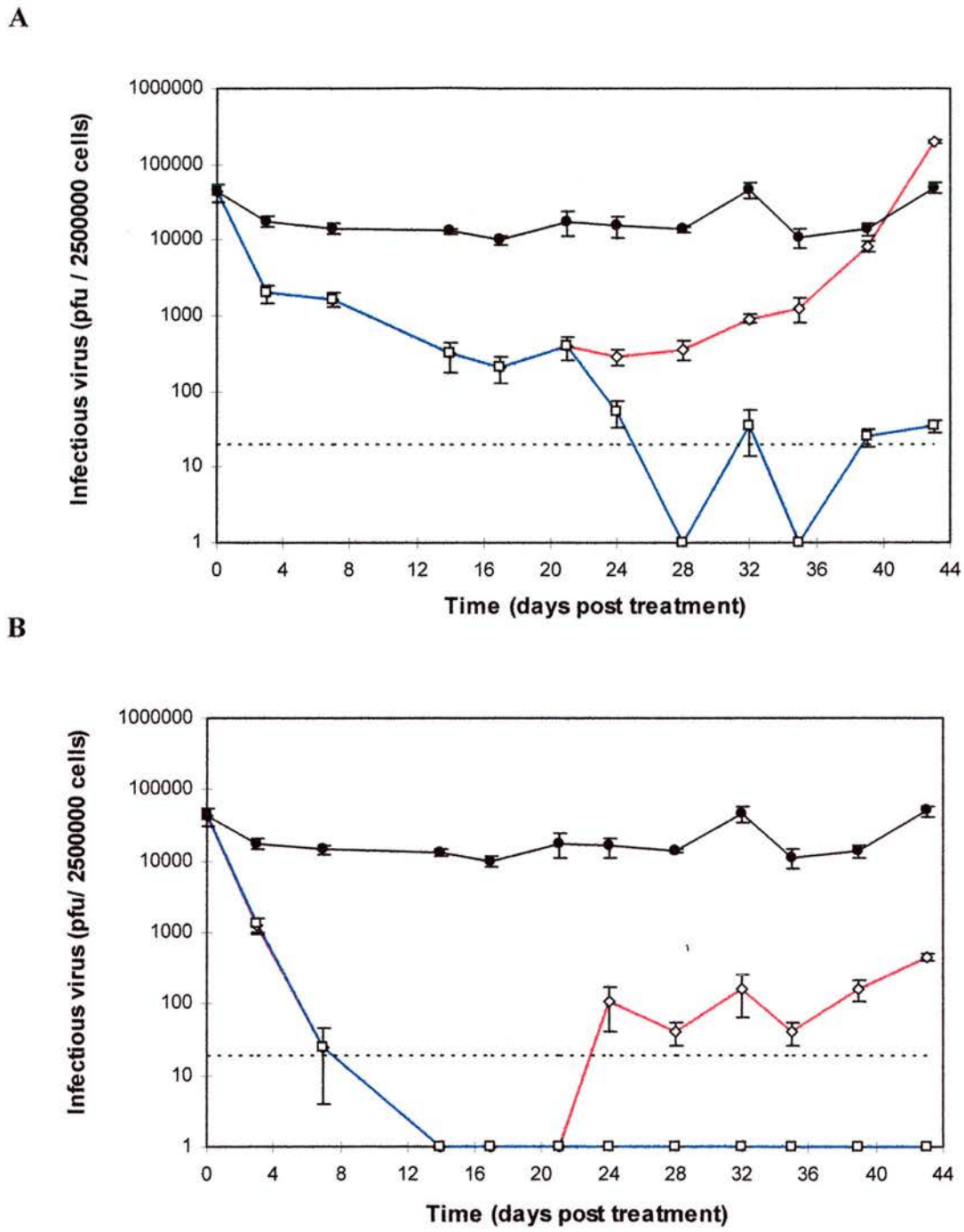


Figure 3.2.1 The levels of productive virus replication that occurred in persistently infected NS0 cultures which had been cultured in the presence of 4'-s-EtdU at 0.2μg/ml (A) and 2μg/ml (B). The persistently infected NS0 cells were either left untreated, continually treated with 4'-s-EtdU (blue) or withdrawn from 4'-s-EtdU after 21 days of treatment (red). The assay limit of detection was 20 pfu per $2.5x10^6$ cells represented by a black dotted line.

Figure 3.2.2

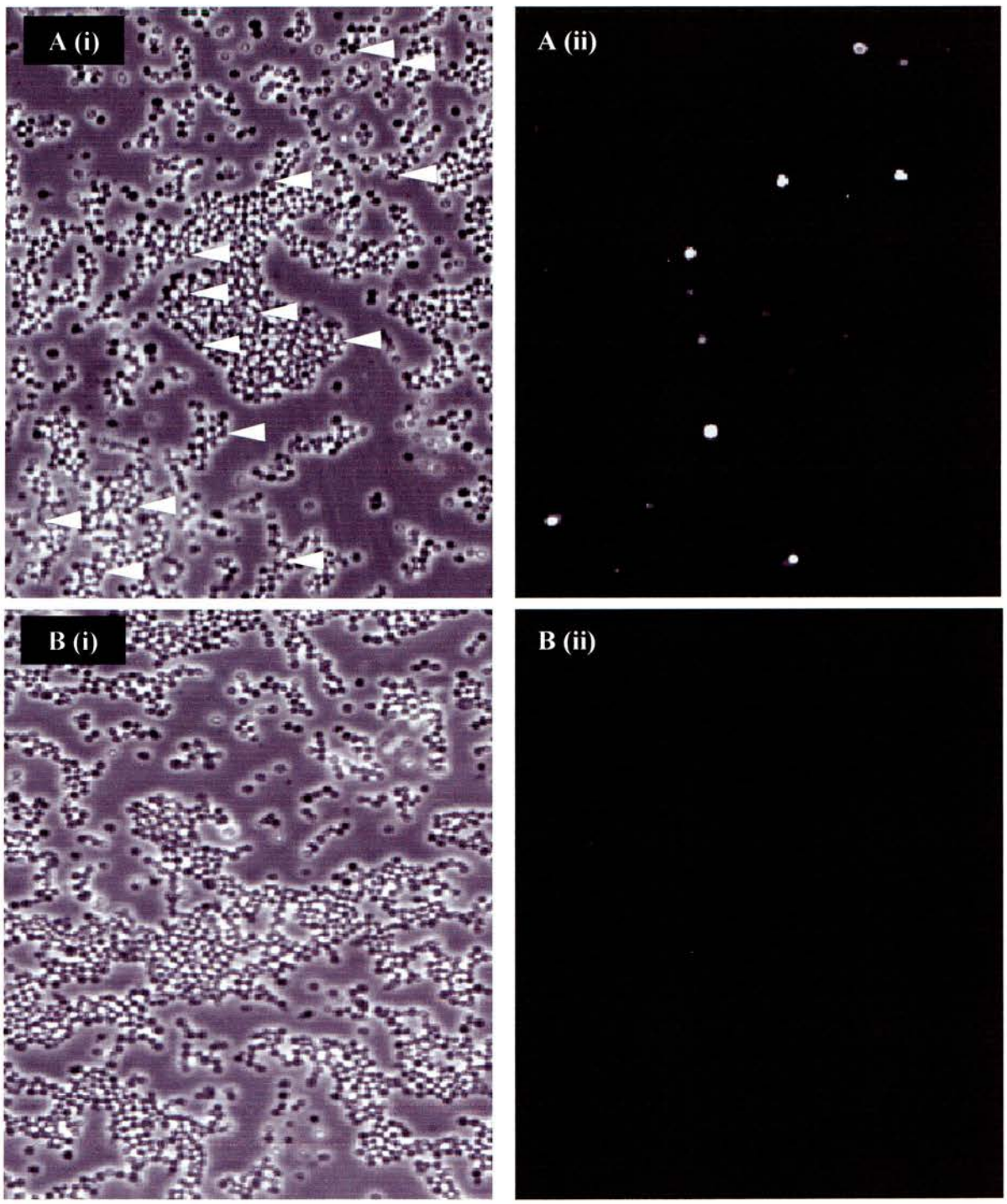


Figure 3.2.2 The abundance of viral antigen in NS0 cultures, persistently infected MHV-68, after 4'-s-EtdU treatment, as determined by immunoflorescent antibody staining. The cells were fixed onto bio-bonded slides and then stained with rabbit anti-MHV-68 hyper-immune sera and pig anti-rabbit FITC. Persistently infected NS0 cells were either treated with 2μg/ml 4'-s-EtdU 10 days (B) or left untreated for the same length of time (A). The cells were viewed by light microscope, using phase contrast (i) and by UV (ii). The viral antigen positive cells show up as white or light grey patches by UV microscopy. The cells these equate to are indicated with white arrow heads.

Figure 3.2.3

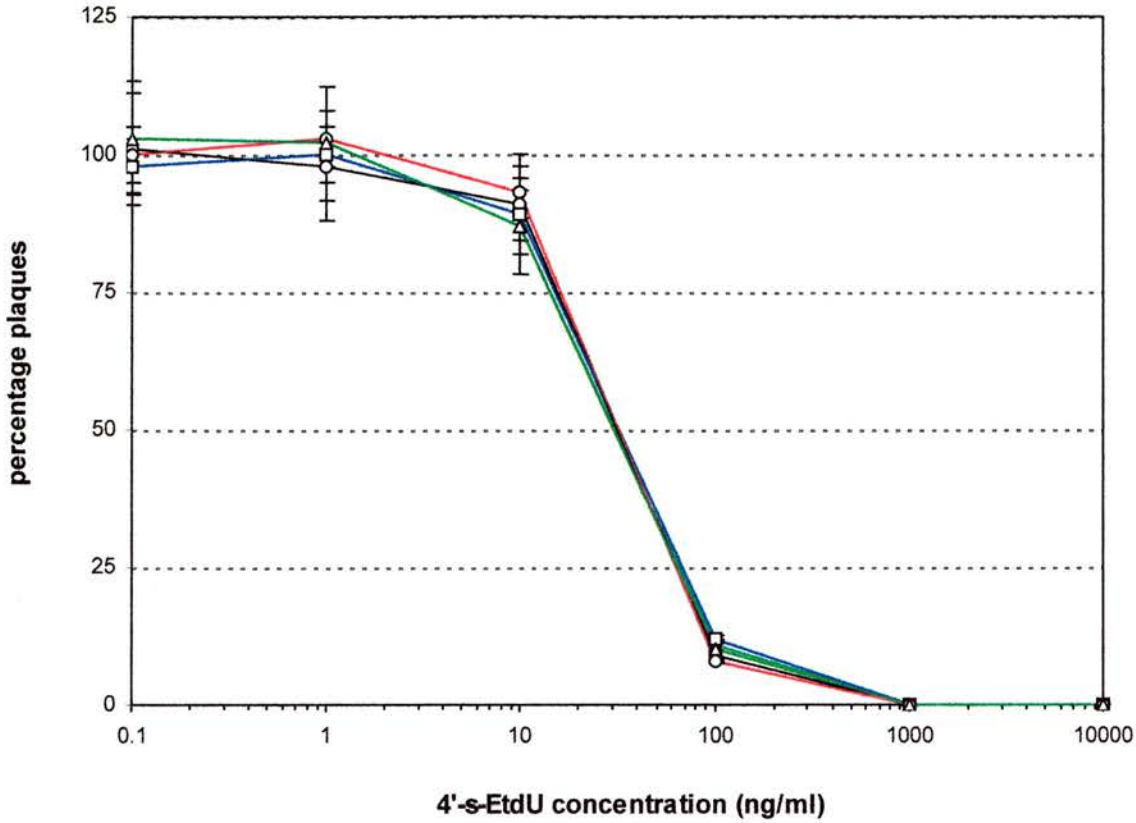


Figure 3.2.3 The plaque reduction profile for MHV-68 isolates derived from persistently infected NS0 cells, as determined by EC_{50} assay. The isolates were derived from either untreated cultures (blue lines and open circles), cultures treated with 0.2 μg/ml 4'-s-EtdU for 47 days (green lines and open triangles), cultures treated with 2 μg/ml 4'-s-EtdU for 47 days (red lines and open diamonds) or the wild type virus stock used originally to generate the persistently infected NS0 line (black lines and solid squares). The dotted lines represent the 100%, 75%, 50% and 25% plaquing values, as determined by the mean number of plaques that occurred in the absence of 4'-s-EtdU.

sensitivity to 4'-s-EtdU were observed between the different virus preparations.

3.2.3 **The inhibitory effects of 4'-s-EtdU on viral persistence in MHV-68 infected NS0 cells**

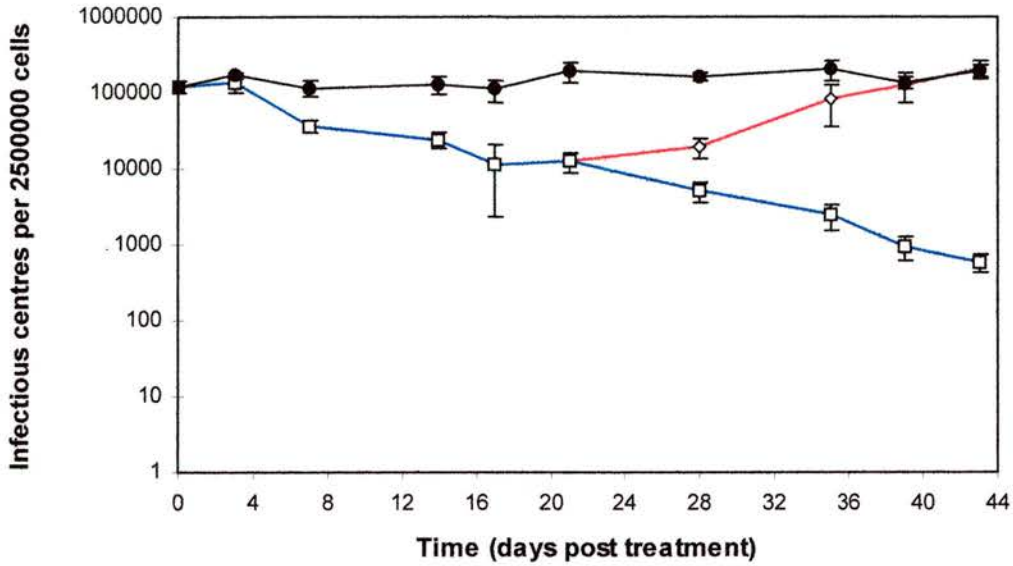
Neither the higher or lower concentration of 4'-s-EtdU could eliminate the virus from the NS0 cultures. At every time point tested, virus could be reactivated from cells by infectious centre assay (*see figure 3.2.4*). However, 4'-s-EtdU treatment of the cultures did significantly reduce the ability of the virus, harboured in the infected cells, to reactivate.

The mean infectious centre titre of the untreated cultures, over the entire experiment was $1.55x10^5$ (+/- $3.4x10^4$) infectious centres per $2.5x10^6$ NS0 cells (On average, 6.2% of the untreated NS0 cells gave rise to infectious centres). Treatment of the persistently infected NS0 cultures with 0.2 μg/ml 4'-s-EtdU reduced the number of infectious centres over the entire time course (*see figure 3.2.4A*). The infectious centre titre decreased 10 fold after 21 days of treatment and over 100 fold after 43 days (0.03% of 2μg/ml 4'-s-EtdU treated NS0 cells gave rise to infectious centres at day 43). On withdrawal of the treatment after 21 days, the infectious centre titre remained at a suppressed level for a week then gradually increased back to the original pre-treatment level. Treatment of the NS0 cultures with 2 μg/ml 4'-s-EtdU likewise suppressed the ability of infected cells to reactivate (*see figure 3.2.4B*). The infectious centre level decreased approximately 10 fold after 7 days of treatment and 1000 fold after 43 days of treatment (0.006% of 2μg/ml 4'-s-EtdU treated NS0 cells gave rise to infectious centres at day 43). After withdrawal of the 2μg/ml 4'-s-EtdU treatment (at day 21) the infectious centre titre remained largely constant and did not return to the pre-treatment titre. The average number of infectious centres observed in the cultures withdrawn from the 2μg/ml 4'-s-EtdU treatment was 3300 (+/- 700) per $2.5x10^6$ NS0 cells (on average 0.13% of cells gave rise to infectious centres).

The suppressed reactivation levels seen in the 2 μg/ml 4'-s-EtdU treated cultures,

Figure 3.2.4

A



B

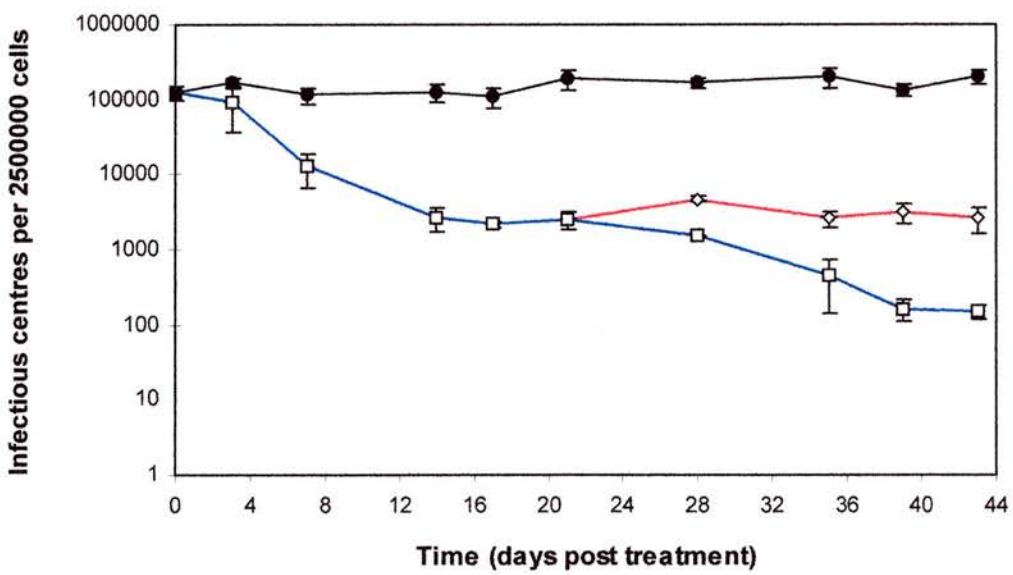


Figure 3.2.4 The levels of virus positive cells, by infectious centre assay, that occurred in persistently infected NS0 cells cultured in the presence of 4'-s-EtdU at 0.2μg/ml (A) and 2μg/ml (B). Persistently infected NS0 cells were either left untreated, continually treated with 4'-s-EtdU (blue) or withdrawn from 4'-s-EtdU after 21 days of treatment (red). The assay limit of detection was 25 infectious centres per $2.5x10^6$ cells.

was not due to a reduced number of cells harbouring viral DNA. There was no significant difference in the proportion of virus positive NS0 cells within the 2 μg/ml 4'-s-EtdU treated culture (at day 21 post treatment) than from the untreated cultures (taken at the same time point). Twenty clonal NS0 cell lines were established from the persistently infected NS0 culture, treated with 2 μg/ml 4'-s-EtdU for 21 days. Of these, 11 harboured viral DNA as determined by first round MHV-68 specific PCR. PCR carried out on the 23 clonal NS0 cell lines were derived from the parallel untreated culture, revealed 15 to be virus DNA positive (*see figure 3.2.5 and 3.2.6*). The decrease in the number of virus positive cell in the treated culture was not significant (using the binomial probability distribution, P=0.23). To determine whether viral DNA present in the clonal cell lines represented viable virus genomes, 6 monoclonal cell lines, all derived from the 4'-s-EtdU treated infected NS0 culture, were retrieved from $N_2(l)$ storage. The cell numbers were expanded over 3 passages and then tested by infectious centre assay. The 2 clonal cell lines which were MHV-68 PCR negative, 10D4 and 13D4, failed to produce infectious centres. The remaining 4 clonal NS0 cell lines, which were all MHV-68 specific PCR positive, 10E9, 11D6, 12E6 and 13E6, all gave rise to approximately 10 infectious centres per 10^6 cells (*see Table 3.3*).

3.2.4 **Protection of MHV-68 infected MGC7 cells with 4'-s-EtdU**

To determine the long-term effects of 4'-s-EtdU on the MHV-68 infection of cell lines which normally only support lytic viral replication, two cell lines, MGC7 and BHK, were chosen. MGC7 cells support relatively slow productive replication of MHV-68, one pfu of MHV-68 takes approximately 6 days to form a visible plaque in a MGC7 monolayer. 4'-s-EtdU pre-treated MGC7 cultures were infected with MHV-68 at a MOI of 1. Initial infection of MGC7 cells, at high multiplicity, lead to cytotoxicity in both the 4'-s-EtdU treated and untreated controls. However, the 4'-s-EtdU treated cells recovered after 3 to 4 days and then assumed normal growth in the 4'-s-EtdU supplemented medium. The infected MGC7 cells treated with a 2μg/ml 4'-s-EtdU medium supplement, could be cultured for over of 2 months. However,

Figure 3.2.5

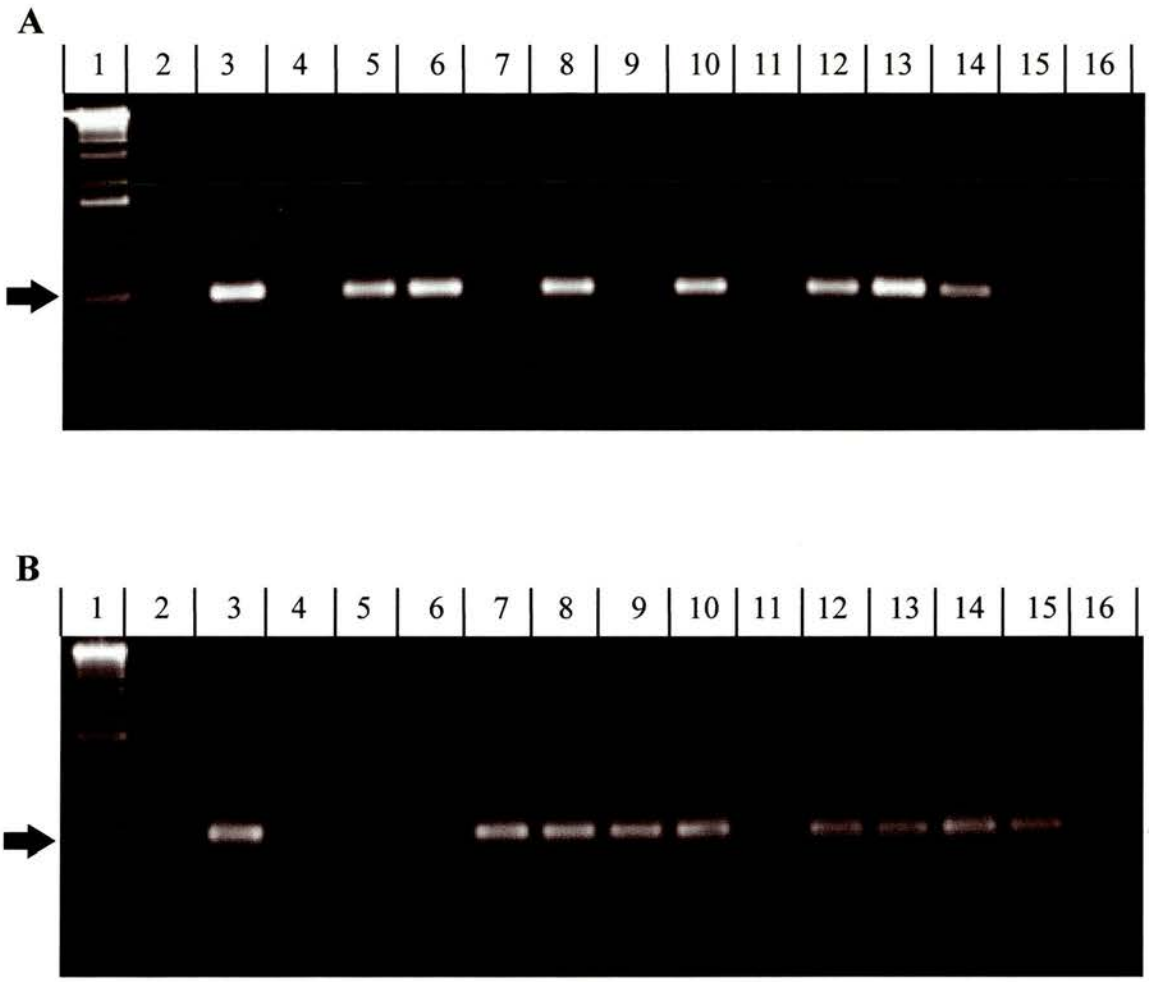


Figure 3.2.5 The first round PCR products amplified from DNA, 250ng, extracted from 23 cloned NS0 cell lines, derived from untreated, persistently MHV-68 infected NS0 cultures (gel A - lanes 5 to 16 and gel B - lanes 5 to 15). PCR was also carried out on 250 ng of DNA extracted from persistently MHV-68 infected (lane 3) and uninfected (lane 4) polyclonal NS0 cultures and distilled water (lane 2) as PCR positive, negative and blank controls, respectively. The Products were visualised using 2% agarose gel electrophoresis (and ethidium bromide staining) and their size determined by comparison to 1 Kb ladder (lane 1). MHV-68 specific PCR bands, at 500 bp, is indicated with black arrows. See table 3.2 for a description of each lane.

Figure 3.2.6

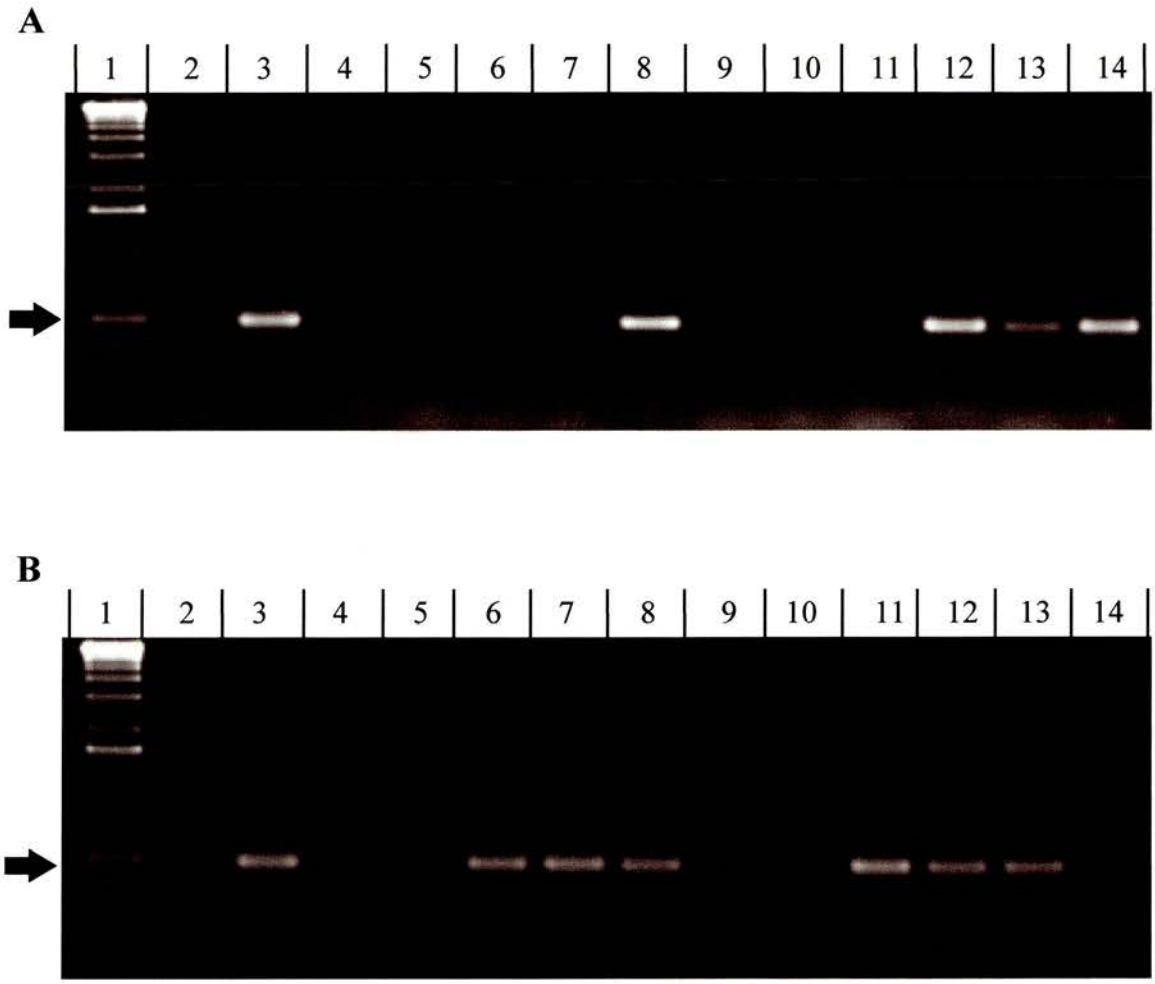


Figure 3.2.6 The first round PCR products amplified from 250ng DNA extracted from the 20 cloned NS0 cell lines, derived from persistently MHV-68 infected NS0 cell cultures after 21 days of 4'-s-EtdU (2 μ g/ml) treatment (lanes 5 to 14 for both gels A and B). PCR was also carried out on 250 ng of DNA extracted from persistently MHV-68 infected (lane 3) and uninfected (lane 4) polyclonal NS0 cultures and distilled water (lane 2) as PCR positive, negative and blank controls, respectively. The products were visualised using 2% agarose gel electrophoresis (and ethidium bromide staining) and their size determined by comparison to 1 Kb ladder (lane 1). MHV-68 specific PCR bands, at 500 bp, is indicated with black arrows. See table 3.2 for a description of each lane.

Table 3.2 **The different cloned NS0 cell lines derived from 4'-s-EtdU treated and untreated MHV-68 infected cultures that underwent the PCR analysis shown in *Figures 3.2.5 and 3.2.6.***

Lane Number	Figure 3.2.5		Figure 3.2.6	
	Gel A	**Gel B**	**Gel A**	**Gel B**
1	1 KB Ladder	1 KB Ladder	1 KB Ladder	1 KB Ladder
2	Blank	Blank	Blank	Blank
3	NS0 (+)	NS0 (+)	NS0 (+)	NS0 (+)
4	NS0 (-)	NS0 (-)	NS0 (-)	NS0 (-)
5	1E4	2B11	10D4	12E7
6	1D2	3C11	10E7	12E5
7	1E2	3H3	10F6	12E6
8	1D5	3A6	10E9	12D7
9	1E5	3D7	11F4	13D8
10	1G5	3H10	11E6	13D4
11	1G8	3B11	11E5	13E6
12	2F1	4G5	11D6	13D8
13	2G5	4D7	11E7	13C9
14	2G7	4A12	11E8	13E9
15	3C3	4D12	-	-
16	2C11	-	-	-

Key: *The 'blank' controls contained no Target DNA. The NS0 (+) and (-) controls refer to PCR carried out on 250 ng of DNA extracted from MHV-68 persistently infected and uninfected NS0 cultures, respectively. The different cloned NS0 lines were named according to the individual well of the 96 well plate they originally grew in. The first digit refers to the plate number. The 96 well plates used to clone the NS0 cells derived from untreated persistently infected NS0 cells were numbered 1 to 4 and those used to clone the 21 day 2μg/ml 4'-s-EtdU treated cultures were labelled 10 to 13. The rows of each 96 well plate were labelled A to H and the columns 1 to 12. Lanes 5 onwards refer to PCR carried out on 250 ng of DNA extracted from the clone specified.*

Figure 3.2.7

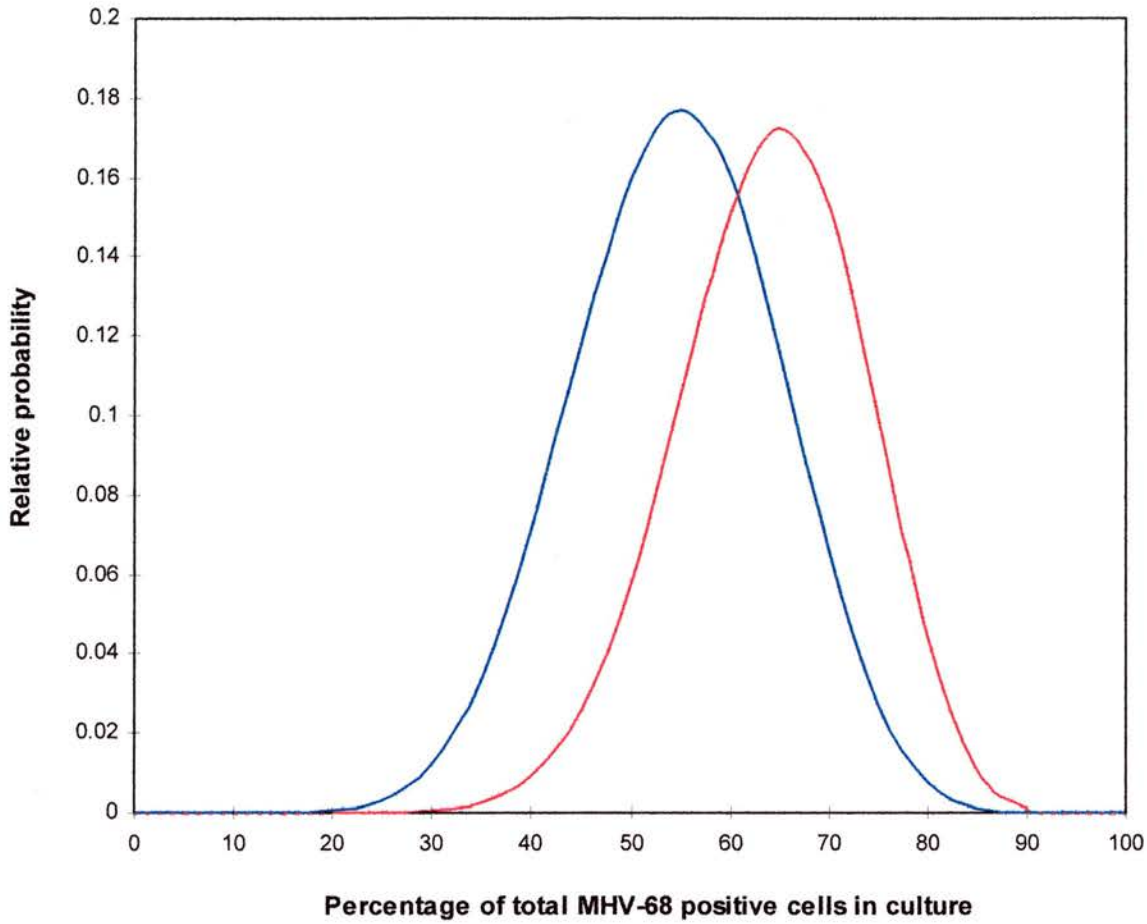


Figure 3.2.7 The possible proportions of virus positive cells that could theoretically be present within the cell populations of persistently MHV-68 infected NS0 cultures, at their relative probability of occurrence. The NS0 cells were either left untreated (red) and treated with 4'-s-EtdU (2μg/ml) for 21 days (Blue). The relative probabilities were determined using the binomial theorem, based on the number of cloned NS0 cell lines that were virus positive by first round, 40 cycle PCR.

Table 3.3 **The relative number of infectious centres produced by different monoclonal cell lines (derived from persistently infected NS0 cultures that had been treated with 4'-s-EtdU at 2μg/ml for 21 days).**

	The number of infectious centres per petri-dish containing					
	10^6 cells	10^5 cells	10^4 cells	10^3 cells	10^2 cells	10^1 cells
NS0(+) [a]	>100	>100	>100	57	5	0
NS0(-) [b]	0	0	0	0	0	0
10E9 [c]	9	1	0	0	0	0
11D6 [c]	12	1	0	0	0	0
12E6 [c]	11	1	0	0	0	0
13E6 [c]	8	1	0	0	0	0
10D4 [d]	0	0	0	0	0	0
13D4 [d]	0	0	0	0	0	0

Key: [a] *Untreated, persistently MHV-68 infected polyclonal NS0 cell line.* [b] *Uninfected polyclonal NS0 cell line.* [c] *MHV-68 PCR positive monoclonal NS0 cell line.* [d] *MHV-68 PCR negative NS0 cell line.*

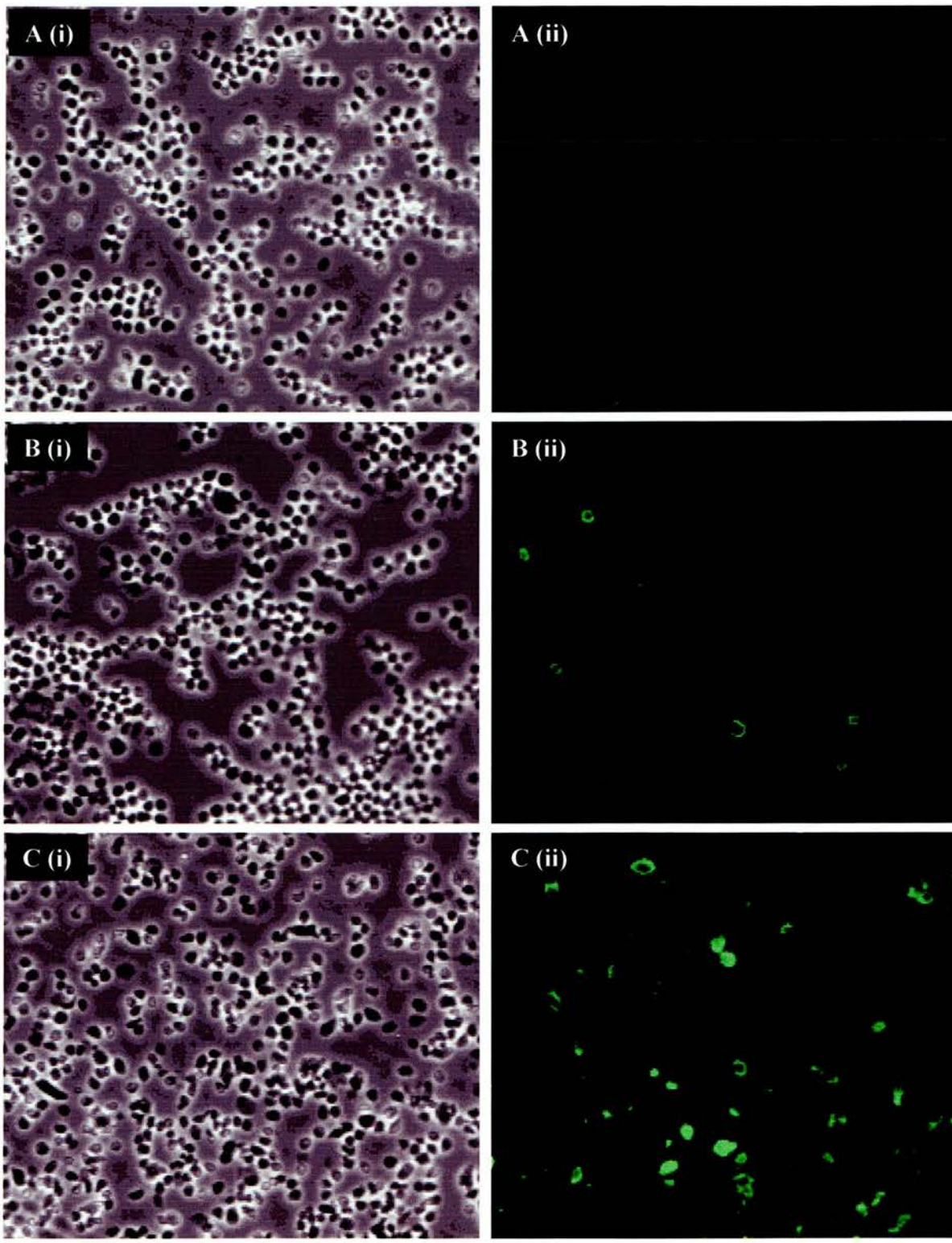
the infected MGC7 cells treated with a 0.2μg/ml 4'-s-EtdU medium supplement died after 2 to 3 weeks.

3.2.5 **The effect of 4'-s-EtdU on viral persistence in MGC7 cells**

MGC7 cells have a doubling time of under 24 hours. To determine whether the input virus was diluted out in the apparent inhibition of productive virus replication, a number of cloned cell lines were derived from an MHV-68 infected MGC7 culture (MOI of 1). The parental culture was infected at an MOI of 1 and cultured in the presence of 2μg/ml 4'-s-EtdU for 32 days. Cells from the culture were then cloned by limiting dilution and cultured in the presents of 2μg/ml 4'-s-EtdU. After 2 weeks, the cloned MGC7 cell outgrowths were withdrawn from the 4'-s-EtdU treatment and cultured for a further 7 days with unsupplemented medium. The medium was then tested for the presence of infectious virus by plaque assay. Of the 12 monoclonal cultures tested, 4 (1/3) gave rise to MHV-68 pfu.

After 2 months, the 2μg/ml 4'-s-EtdU treatment was withdrawn from persistently infected MGC7 cultures and productive replication allowed to reinitiate. The treated cultures were virus antigen negative at the point of withdrawal, as determined by fluorescent immunocytochemistry. The staining of the MGC-7 cells, was carried out in parallel, using 2 different seras, rabbit anti-MHV-68 hyper-immune serum and late time point (day 60 post infection) mouse serum. Two days after the 4'-s-EtdU was withdrawn, a small proportion of the MGC7 cells (3.5%) became viral antigen positive. This increased to approximately 40% by 4 days and was over 95% by day 6. Up to day 6, the fluorescent signal observed in the stained cells was predominately of low intensity. However, by day 8, the staining was predominately of high intensity. All the cells were virus antigen positive by this time and the cells were starting to form large clumps. All the cells were dead by day 10 (*see figure 3.3.1*). No significant differences were observed using the 2 different sera.

Figure 3.3.1



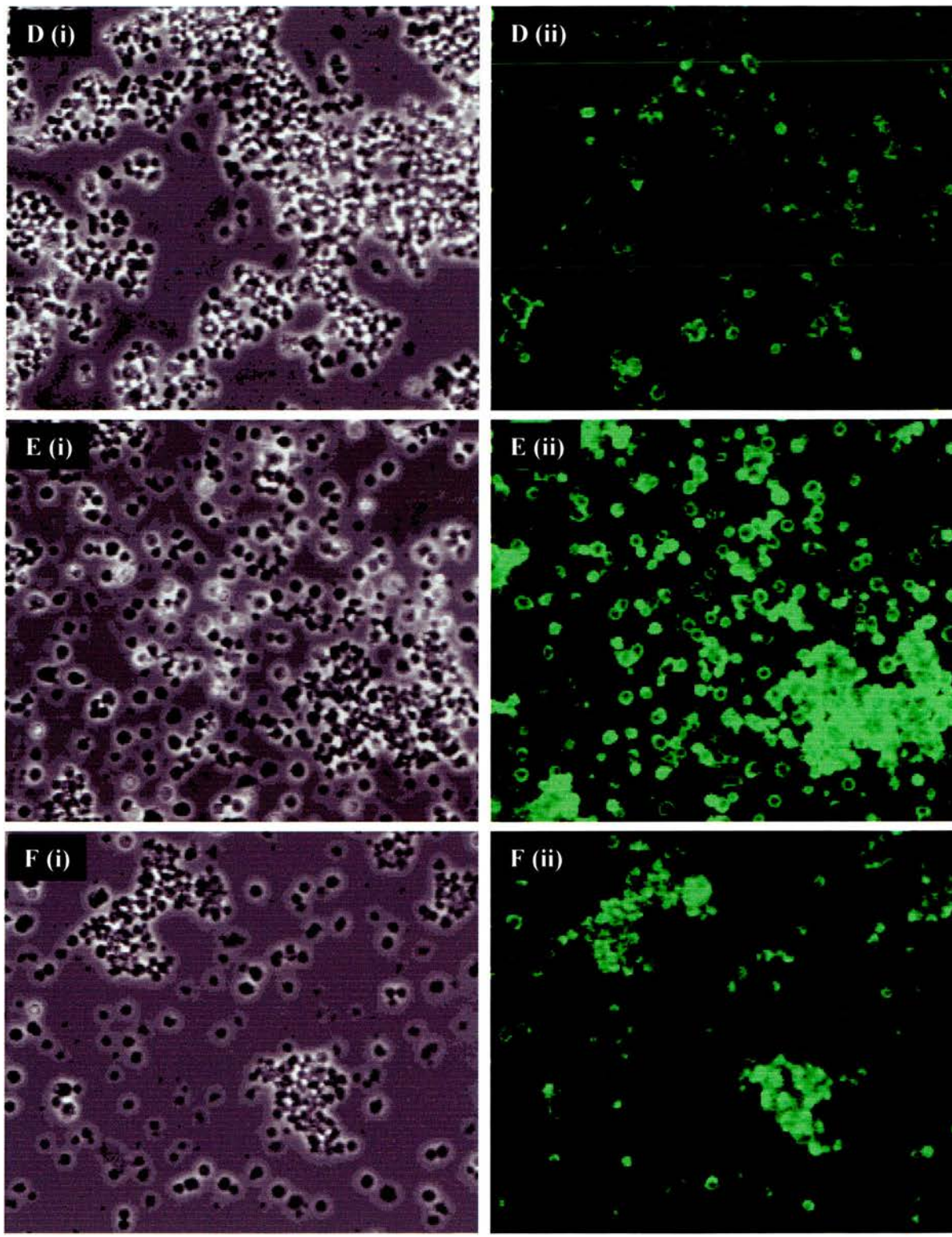


Figure 3.3.1 shows persistently MHV-68 infected MGC7 cells 0 days (A), 2 days (B), 4 days (C), 6 days (D), 8 days (E) and 10 days (F) after withdrawal of 2μg/ml 4'-s-EtdU treatment. The cells were fixed onto bio-bonded slides and stained with rabbit anti-MHV-68 hyper-immune serum and FITC conjugated swine anti-rabbit serum. The slides were viewed under phase contrast light microscopy (i) and UV microscopy (ii). Cells positive for productive viral antigen appear fluorescent green by UV microscopy.

3.2.6 **The cloning and characterisation of 4'-s-EtdU resistant virus generated spontaneously from 4'-s-EtdU treated, MHV-68 infected, MGC7 cells**

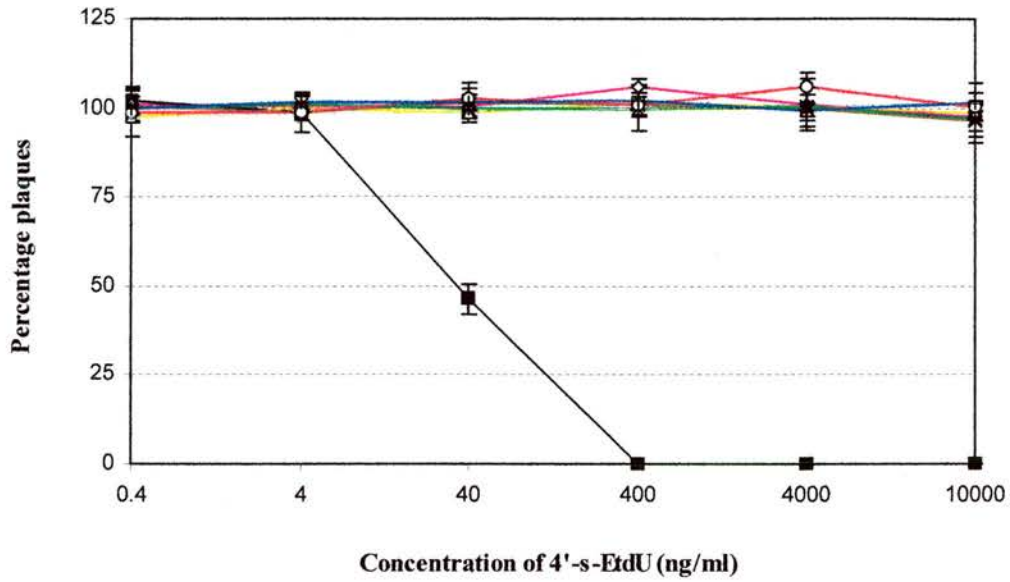
Persistently infected MGC7 cultures, cultured for long periods of time (in excess of 2 months) spontaneously went into crisis. Culture medium from one such flask was shown, by plaque assay to contain infectious virus that would form plaques in the presence of 2μg/ml 4'-s-EtdU (*data not shown*). The 4'-s-EtdU resistant virus, contained within the culture medium underwent three consecutive rounds of limiting dilution plaque purification. This gave rise to 6 cloned 4'-s-EtdU resistant virus isolates. The isolates were named MGC7 derived 4'-s-EtdU resistant virus (MERV) 34/22/41, 34/22/44, 43/62/67, 43/62/72, 43/68/89 and 43/68/92 (the numerical suffix denotes the well numbers selected during the cloning). All six MERV clones were unaffected, with respect to *in vitro* plaquing efficiency, by 4'-s-EtdU up to 10μg/ml. However, all remained sensitive to the inhibitory effects of ACV (for plaque reduction profiles see *figure 3.3.2*).

3.2.7 **The effect of 4'-s-EtdU on the MHV-68 infection of BHK cells**

BHK cells support rapid productive viral replication. One pfu of MHV-68 takes approximately 4 days to form a visible plaque (by light microscopy) in a BHK monolayer. Infection of 4'-s-EtdU pre-treated BHK cells, at a MOI of 4, resulted in all the cells in the culture dying. However, BHK cells could clearly be protected for short periods of time (4 days) at low MOI, as demonstrated by EC_{50} assay (*see figure 3.2*). An experiment was therefore set up test the ability of 4'-s-EtdU, at 2μg/ml, to protect BHK cultures infected at varied MOI. The 4'-s-EtdU treatment did not protect the BHK cultures at 4 pfu per cell (*see figure 3.4.1*) and only partially protected cultures at 0.4 pfu per cell (*see figure 3.4.2*). Surviving BHK cells, infected at a 0.4 pfu per cell, underwent limited expansion, with respect to the number of viable cells in the culture. However, the cultures did not fully recover, with respect to both viability and growth rate. The 4'-s-EtdU treatment protected cultures infected at an MOI of 0.04 pfu per cell or lower (*see figures 3.4.3, 3.4.4 and*

Figure 3.3.2

A



B

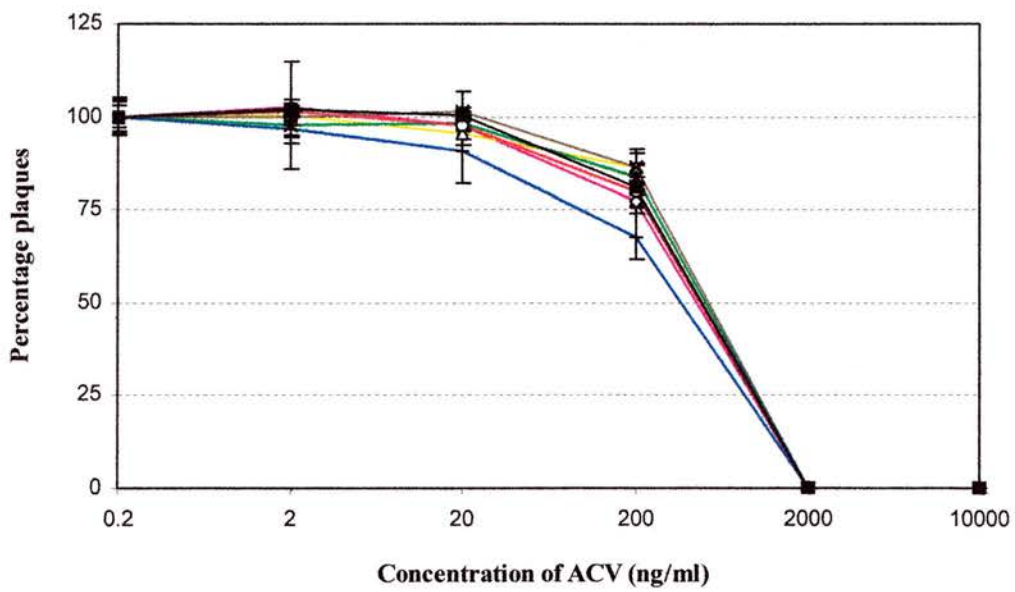


Figure 3.3.2 The plaque reduction profiles for the 4'-s-EtdU resistant MHV-68 clones MERV 34/22/41 (open diamonds with pink lines), 34/22/44 (open triangles with yellow lines),43/62/67 (diagonal crosses with brown lines), 43/62/72 (asterisks with green lines), 43/68/89 (open circles with red lines) and 43/68/92 (horizontal/vertical crosses with blue lines) against both 4'-s-EtdU (A) and ACV (B). These were compared with wild type MHV-68 (solid squares with black lines). The assays were all done in parallel and the percentage plaque values derived from the plaquing efficiency of untreated controls. The dotted lines represent the 100%, 75%, 50% and 25% plaquing efficiency values.

3.4.5). BHK cells infected with $4x10^{-2}$ to $4x10^{-6}$ pfu per cell and cultured with 2μg/ml 4'-s-EtdU, were indistinguishable from uninfected BHK cells, derived from the same parental culture, with respect to both viability and growth rates (*see figure 3.4.6*). However, protection mediated by the 4'-s-EtdU treatment tended to be transient, since spontaneous lytic replication of 4'-s-EtdU resistant virus generally occurred after approximately 12 days, except for the cultures infected at $4x10^{-5}$ to $4x10^{-6}$ pfu per cell. Virus resistant to 4'-s-EtdU first became detectable by the appearance of discrete plaques in an otherwise healthy monolayer. Over a period of 2 to 3 days the plaques spread though out the monolayer, resulting in the death of all BHK cell present. MHV-68 was not lost from any of the infected cultures even those infected with the lowest MOI. BHK cells infected at a MOI of $4x10^{-5}$ and $4x10^{-6}$ were withdrawn from treatment after 20 days of culture. Viral plaques became detectable, by light microscopy, after 4 to 6 days, respectively and the monolayers completely destroyed by 10 days (*data not shown*).

Figure 3.4.1

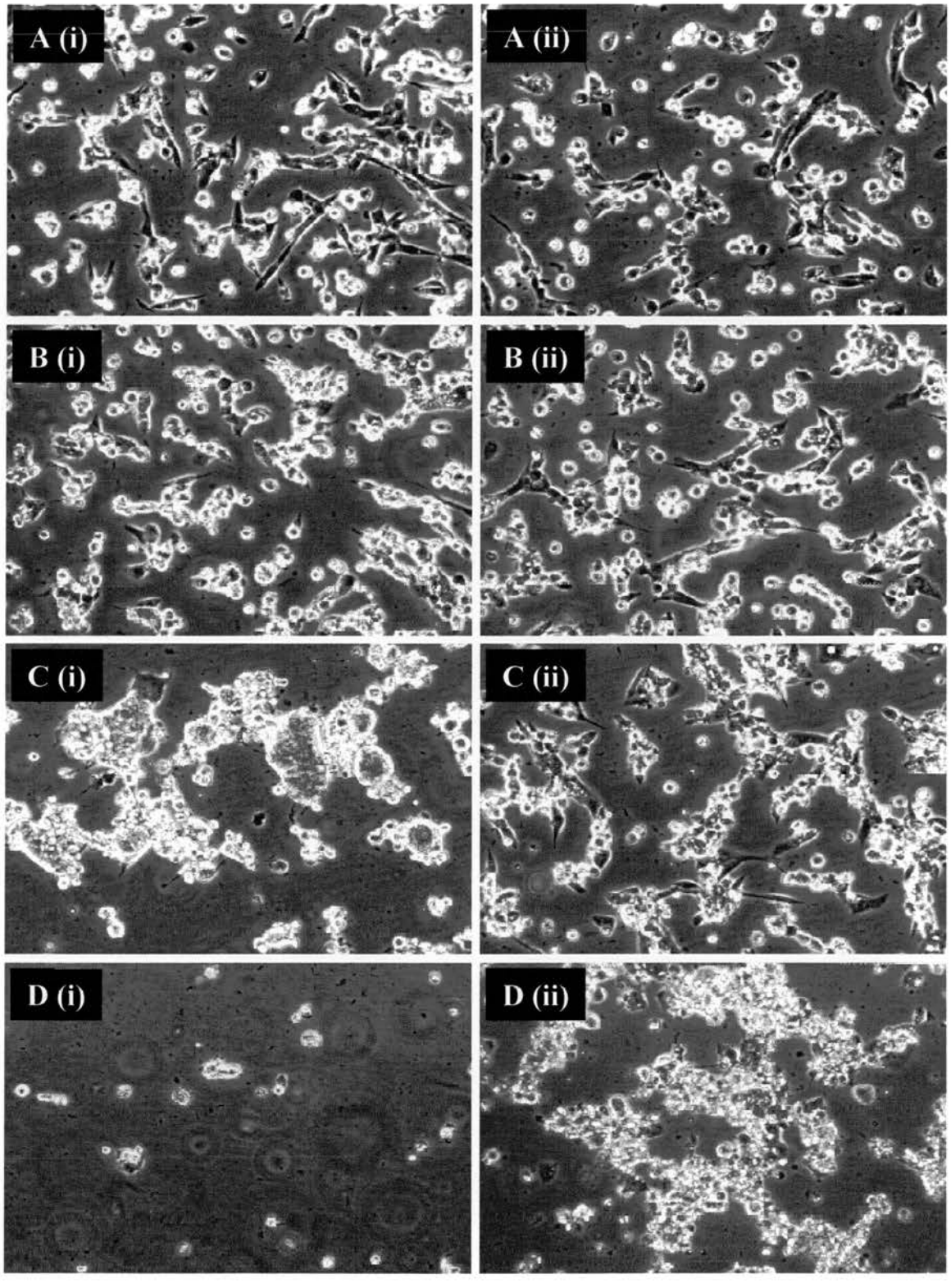


Figure 3.4.1 The effects of MHV-68 infection, MOI 4, on BHK cells, grown using ECT10 growth medium which was either left unsupplemented (i) or supplemented with 4'-s-EtdU at 2μg/ml (ii). The cells were initially seeded at 5x10^6 cells per T40 flask and were passaged as and when required. The cultures were photographed in situ, on day 1 (A), 3 (B), 5 (C) and 10 (D) post infection, using a light microscope, under phase contrast (x20).

Figure 3.4.2

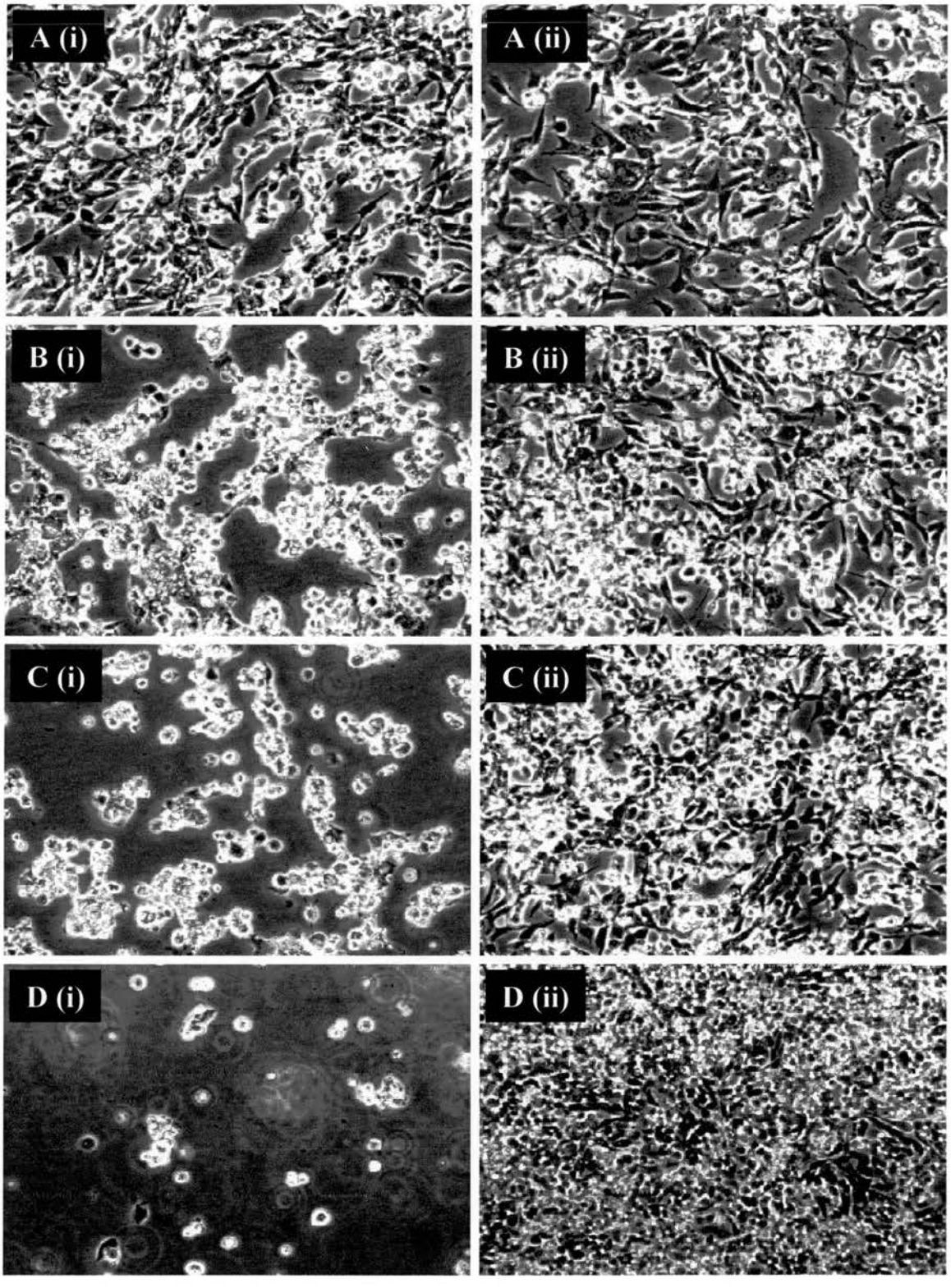


Figure 3.4.2 The effects of MHV-68 infection, MOI of 0.4, on BHK cells, grown using ECT10 growth medium which was either left unsupplemented (i) or supplemented with 4'-s-EtdU at 2μg/ml (ii). The cells were initially seeded at $5x10^6$ cells per T40 flask and were passageas and when required. The cultures were photographed in situ, on day 1 (A), 3 (B), 5 (C) and 10 (D) post infection, using a light microscope, under phase contrast (x20).

Figure 3.4.3

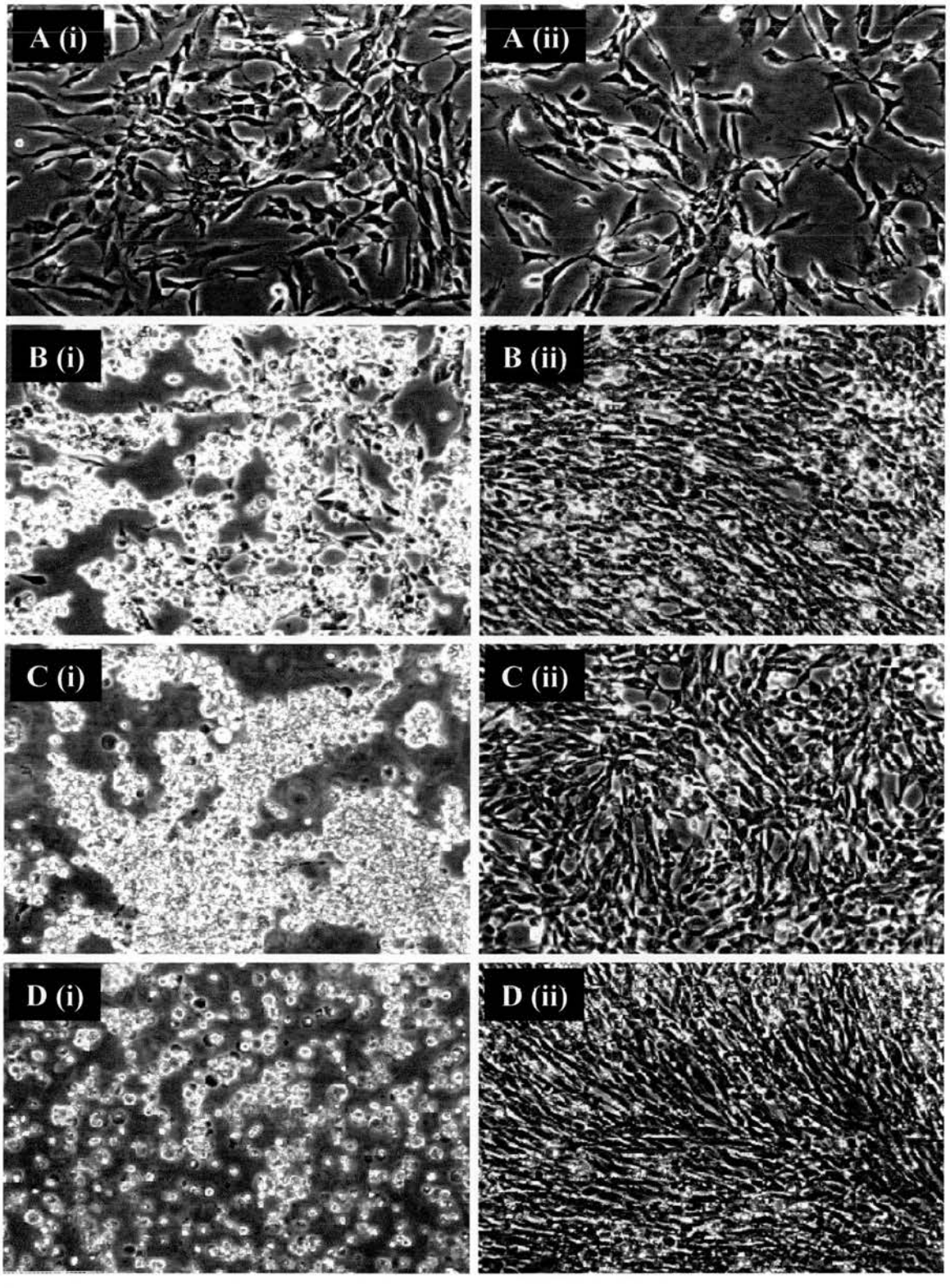


Figure 3.4.3 The effects of MHV-68 infection, MOI of $4x10^{-2}$ on BHK cells, grown using ECT10 growth medium which was either left unsupplemented (i) or supplemented with 4'-s-EtdU at 2μg/ml (ii). The cells were initially seeded at $5x10^{6}$ cells per T40 flask and were passageas and when required. The cultures were photographed in situ, on day 1 (A), 3 (B), 5 (C) and 10 (D) post infection, using a light microscope, under phase contrast (x20).

Figure 3.4.4

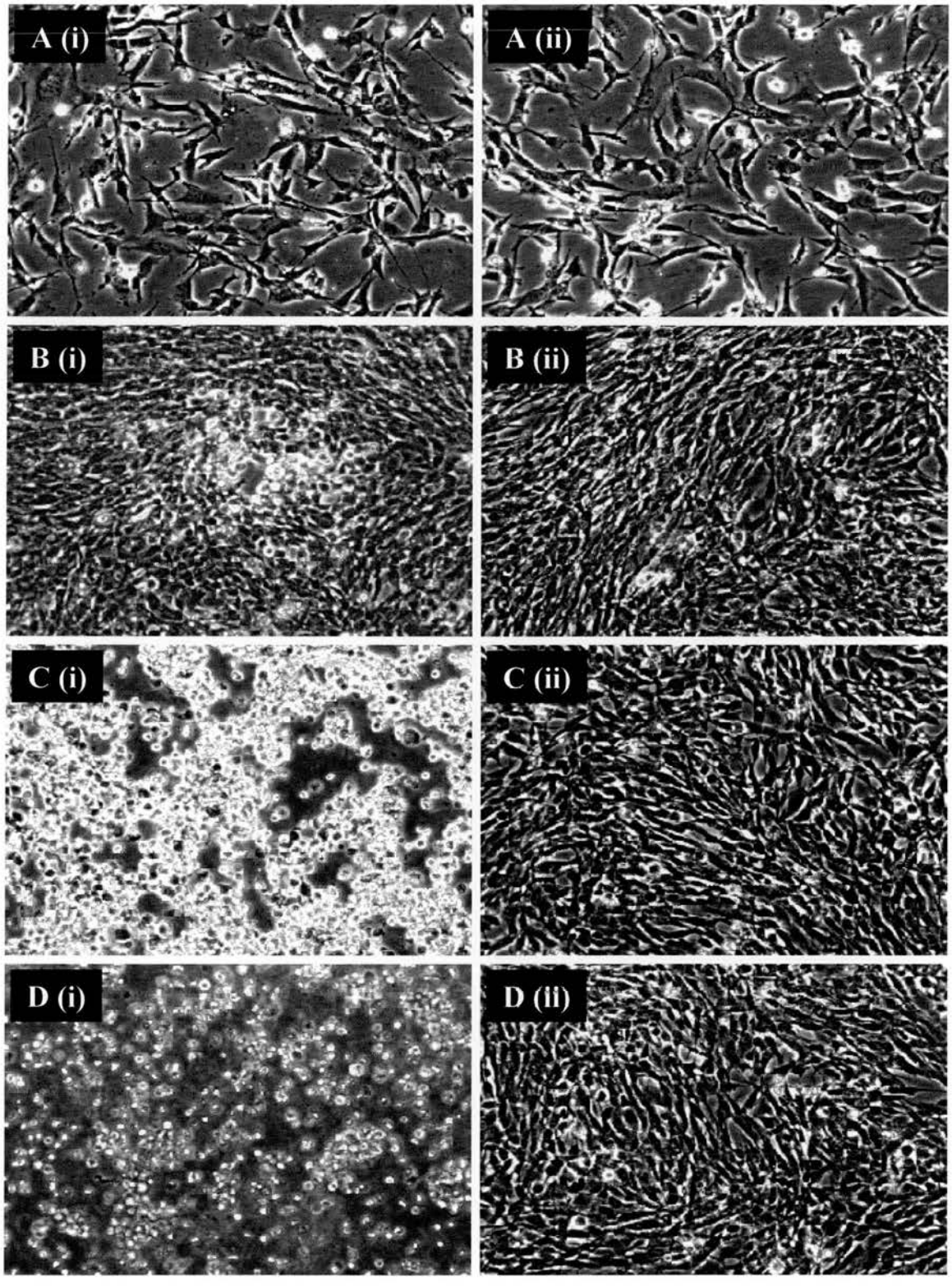


Figure 3.4.4 The effects of MHV-68 infection, MOI of 4x10 $^{-3}$ on BHK cells, grown using ECT10 growth medium which was either left unsupplemented (i) or supplemented with 4'-s-EtdU at 2μg/ml (ii). The cells were initially seeded at 5x10^{6} cells per T40 flask and were passageas and when required. The cultures were photographed in situ, on day 1 (A), 3 (B), 5 (C) and 10 (D) post infection, using a light microscope, under phase contrast (x20).

Figure 3.4.5

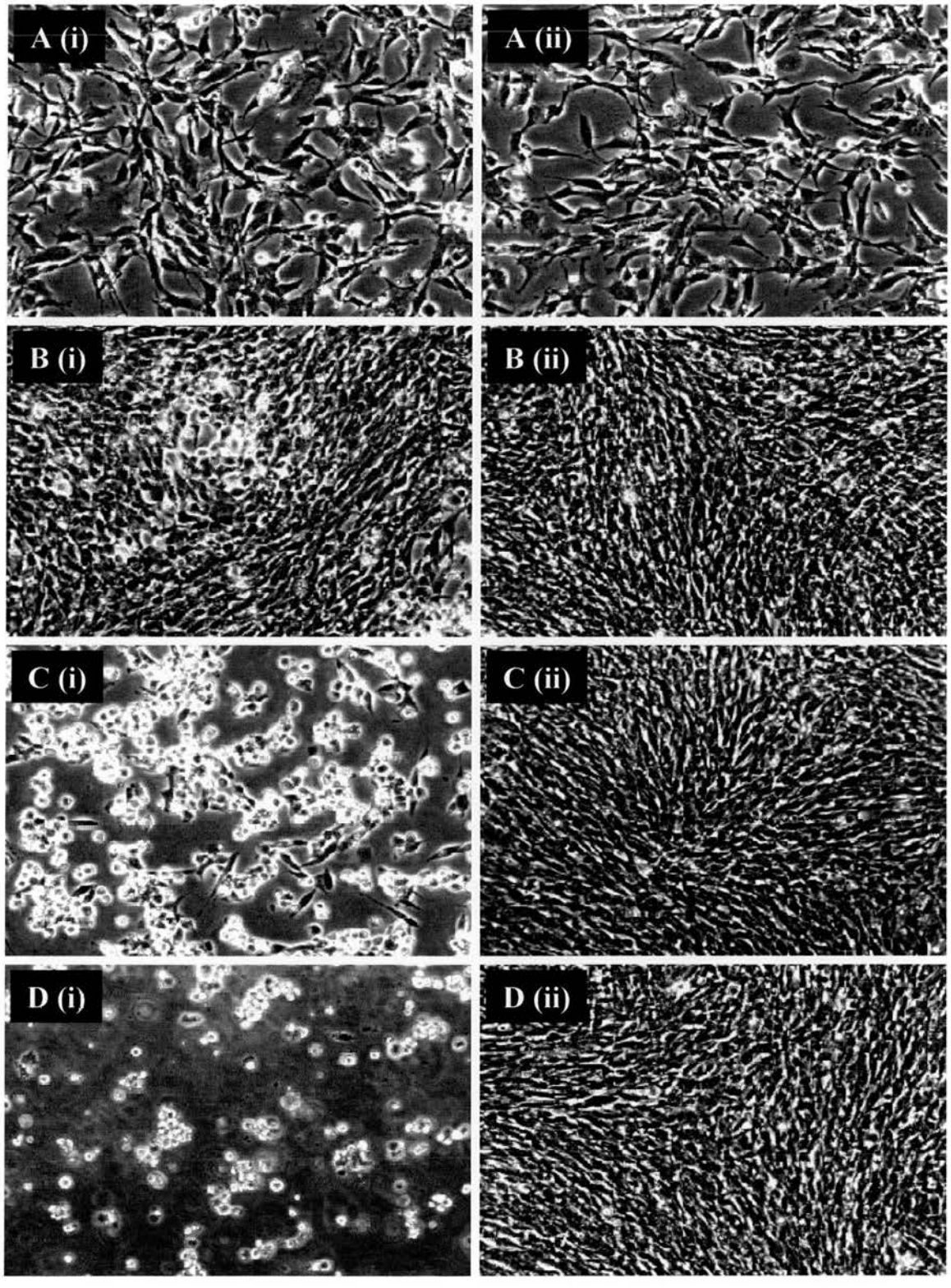


Figure 3.4.5 The effects of MHV-68 infection, MOI of $4x10^{-4}$ on BHK cells, grown using ECT10 growth medium which was either left unsupplemented (i) or supplemented with 4'-s-EtdU at 2μg/ml (ii). The cells were initially seeded at $5x10^{6}$ cells per T40 flask and were passageas and when required. The cultures were photographed in situ, on day 1 (A), 3 (B), 5 (C) and 10 (D) post infection, using a light microscope, under phase contrast (x20).

Figure 3.4.6

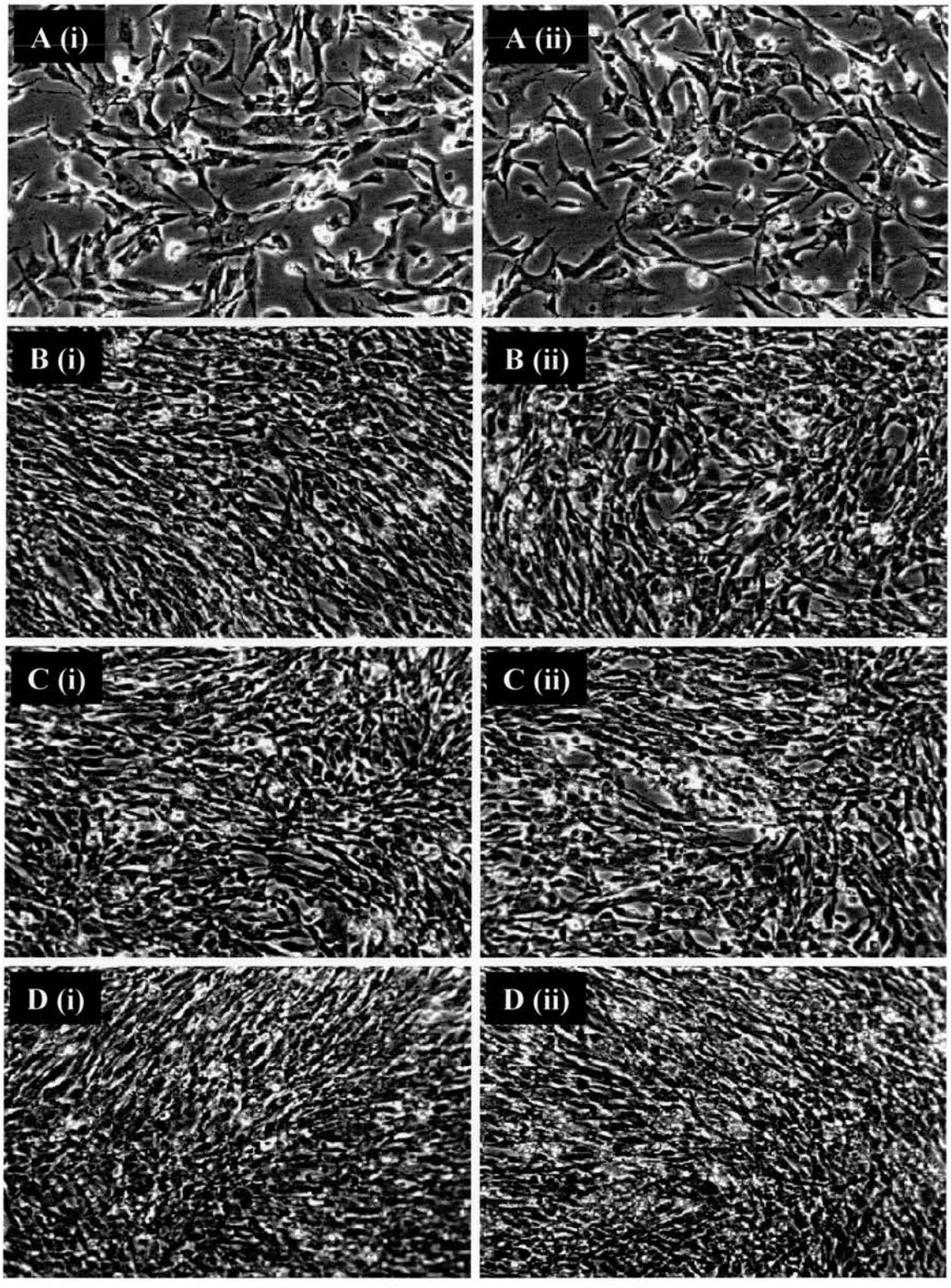


Figure 3.4.6 Uninfected control BHK cells grown on ECT10 growth medium which was either left unsupplemented (i) or supplemented with 4'-s-EtdU at 2μg/ml (ii). The cells were initially seeded at $5x10^6$ cells per T40 flask and were passageas and when required. The cultures were photographed in situ, on day 1 (A), 3 (B), 5 (C) and 10 (D) post mock infection, using a light microscope, under phase contrast (x20).

3.2.8 The effectiveness of 4'-s-EtdU as an inhibitor of MHV-68 *in vitro*.

In vitro, 4'-s-EtdU was shown to be at least 10 times more potent at inhibiting the lytic replication of MHV-68 than ACV, in BHK cells. The EC_{50} of 4'-s-EtdU was evaluated to be 35 ng/ml, which is equivalent to a 0.13 μM solution. This compares to ACV, which had an EC_{50}, under parallel conditions, of 450ng/ml (2μM). The ACV value is higher than has been previously reported (Sunil-chandra, *et al*, 1993) but is within 1 order of magnitude. A possible explanation for the difference between the two EC_{50} values is that with the study carried out by Sunil-Chandra, the EC_{50} assays were carry out using ACV kept in suspension. In this study, the anti-virals were dissolved, by heating to 60°C, and then filter-sterilised, prior to use. Although this may account for the difference between the 2 different ACV EC_{50} values, it is unlikely to have affected the relative difference in potency between ACV and 4'-s-EtdU, since both were tested and treated in exactly the same manner.

3.2.9 Viral persistence in NS0 cells

MHV-68 is B-cell tropic and establishes latency in these cells. NS0 cells are a transformed myeloid cell of B-cell origin. The persistent infection established by MHV-68 in NS0 cells is believed to be an *in vitro* model for viral latency coupled with spontaneous reactivation. The evidence for the establishment of viral latency comes from the fact that the cultures contain episomal DNA as detected by Gardella gel electrophoresis. Virus can be reactivated from the cells be co-cultivation assay, despite the majority of the cells being virus antigen negative, as detected by polyclonal anti-MHV-68 hyper-immune serum (Sunil-Chandra *et al*, 1993). By studying the effects of anti-virals on infected cell lines which supports both latent as well as limited productive viral replication, it is possible to gain insights into how long-term treatment can affect viral persistence and the balance between viral latency and reactivation.

Previous studies have shown that after the initial infection of NS0 cells, high tires of cell free virus are present in the culture medium for the first 2 to 3 days post infection. The cell free virus titres then decrease to below the cell associated virus levels, which remained largely constant (Sunil-Chandra *et al*, 1993). So as to allow the persistently infected NS0 cells to achieve equilibrium, with respect to virus load and reactivation levels, 4'-s-EtdU treatment did not start at day 0 post infection. Instead the MHV-68 infected NS0 cells were passage 3 times, frozen in liquid nitrogen, retrieved and then passaged a further 3 time prior to treatment initiation. Thr.ough out the experimental time-course, the non-4'-s-EtdU treated MHV-68 infected cultures remained largely constant with respect to numbers of cells undergoing reactivation, as determined by both co-cultivation and infectious virus. The levels of cell associated infectious virus present within the culture at any one instant, was generally at least 10 times less, than the amount of infectious centres that could be generated by 5 day co-cultivation assay. The levels of infectious virus present in the NS0 cultures ranged from $1 - 2 \times 10^4$ pfu per 10^6 cells and the numbers of cells virus antigen positive by immunocytochemistry rarely exceeded 2%. These results are indicative of a persistently infected culture undergoing a low level of continual, but constant, virus reactivation.

3.3.1 **The effects of 4'-s-EtdU on productive virus replication in NS0 cells**

Treatment of the persistently infected NS0 cell cultures with 2μg/ml 4'-s-EtdU, readily eliminated productive viral replication and late gene expression, from the cells. By 14 days post treatment, no infectious virus could be detected by direct plaque assay and no virus antigen could be detected by immunocytochemistry. However, 4'-s-EtdU treatment did not eliminate the virus from the cultures, since virus could always be reactivated from cells by co-cultivation assay. This is largely consistent with the findings from a previous study, determining the effects of ACV treatment on persistently infected NS0 cells (Sunil-Chandra *et al*, 1993). Treatment of persistently infected NS0 cells with ACV at 25μg/ml, resulted in the elimination

of detectable infectious virus from the cells, by day 32 post treatment. The ACV treatment also failed to eliminate latent virus from the culture since infectious centres remained detectable. After 10 days of treatment the level of linear viral genome harboured within the cells was significantly reduced, as determined by Gardella gel electrophoresis, in line with the decrease in productive virus replication, as determined by infectious virus assay. The Gardella gel electrophoresis also showed the level of episomal DNA contained within the cells was only minimally decreased by treatment (Sunil-Chandra, *et al*, 1993). The relative potency of ACV, as compared to 4'-s-EtdU, in the elimination of infectious virus from the persistently infected NS0 cultures, is in agreement with the differences in potency observed by EC_{50} assay. ACV at 25 μg/ml, took longer to eliminate the productive MHV-68 replication occurring in the NS0 cultures than 4'-s-EtdU at over a 10 times lower concentration.

Further key differences exist between the reported effects of ACV treatment of infected NS0 cells and the effects of 4'-s-EtdU, reported in the study. The ACV treatment failed to eliminate virus antigen expression, as determined by immunohistochemistry. Where as 4'-s-EtdU treatment at 2μg/ml eliminated virus antigen expression from both weekly and strongly staining infected cell populations. This is again indicative of the greater potency of 4'-s-EtdU in the inhibition of MHV-68. Although a FITC conjugated secondary antibody was used in this study, instead of a horseradish peroxidase based detection system, the primary antibody was the same for both studies. Therefore the detection levels for both immunostaining approaches should have been similar.

The overall pattern, with respect to productive virus replication and latency reactivation, was similar with both 0.2μg/ml and 2μg/ml 4'-s-EtdU treatments of persistently infected NS0 cells. Both concentrations of 4'-s-EtdU reduced the levels of cell associated infectious virus, present within the cultures. Further more, on withdrawal, after 21 days, the levels of productive virus replication in the cell cultures, once more increased. Although treatment of the cultures with 0.2μg/ml 4'-

s-EtdU reduce the levels of cell associated infectious virus 1000 fold, unlike the 4'-s-EtdU treatment at 2μg/ml, it failed to eliminate all productive virus replication. By EC_{50} assay, 0.2μg/ml 4'-s-EtdU was sufficient to prevent MHV-68 forming plaques in BHK cells. However, the concentration was clearly insufficient to inhibit the productive replication of MHV-68 entirely.

3.3.2 **The effect of 4'-s-EtdU on viral reactivation from persistently infected NS0 cells**

At both concentrations of 4'-s-EtdU, there was a suppression of the ability of latently infected cells to reactivate, as determined by infectious centre assay. The suppression was more marked with the higher dose of 4'-s-EtdU but remained significant with the lower dose. The apparent suppressed latency phenotype was not due to the carry over of 4'-s-EtdU into the infectious centre assay, since it was a phenotype that was acquired gradually over a period of weeks. The suppression was only partial since neither treatment dose could entirely eliminate cells capable of reactivation.

In part, the suppressed latency phenotype could be due to inhibition of productive virus replication prior to the infectious centre assay taking place. Immunostaining of the persistently infected NS0 cells revealed 2% of the cells to be virus antigen positive. Cloning the cells revealed approximately 65% of the total cells contained viral DNA, as determined by PCR. On the assumption that all viral DNA containing cells harbour viable virus genomes, (an assumption based on the fact that virus could be reactivated from all the PCR positive NS0 clones tested) only a small proportion, approximately 3%, of the virus infected cells expressed detectable levels of virus antigen. It is therefore not unreasonable to assume that the rabbit anti-MHV-68 hyper-immune serum, could not pick up latently infected cells. Of the 2% of cells that did stain, approximately 30% stained brightly and hence probably contained cell-associated infectious virus. The remaining cells, approximately 70%, of the 2% that stained, stained only weakly. These cells may not have contained viable

progeny but were clearly already in the process of reactivation. This would mean between 30 to 40% of the cells that would have given rise to an infectious centre by co-cultivation assay, would have already started to reactivate prior to the assay being performed. It is therefore reasonable to assume that inhibition or reduction of productive replication prior to the assay being performed, would reduce the number of resultant infectious centres by up to 40%.

Reduction or elimination of productive virus replication within the NS0 cell cultures, even if the virus pfu were spread optimally though out the culture, could only account for a small proportion of the decrease in infectious centre titre. This is due to the total infectious virus titre always being a fraction of the total infectious centres, observed per culture. There were also fundamental differences in the dynamics of the rates of decrease, seen in the infectious virus and infectious centre titres. This was particularly apparent with the infected NS0 cell cultures treated with 2μg/ml 4'-s-EtdU. The cell associated infectious virus titre was virtually eliminated after 7 days of treatment, and undetectable from day 14. In contrast, the infectious centre titre decreased at a relatively constant rate, over the entire course of the treatment.

A more likely explanation for the decreased levels of virus reactivation would be that the infected cells with a transcriptional environment less suited for stable virus latency, are selectively growth inhibited or killed in the presence of 4'-s-EtdU. This could have been to the reactivation associated up regulation of viral TK which would activates the 4'-s-EtdU present in the cell and inhibit cellular DNA synthesis. Virus reactivation is a sporadic event that occurs in only a small proportion of infected NS0 cells, at any one time. The gradual depletion of the cells less capable of harbouring 'tightly' latent virus, would probably go undetected by trypan blue viability testing of the cell culture. This explanation fits well with the dynamics of the suppression of virus reactivation, observed over the time course. The decrease in infectious centres, followed the general pattern of exponential decay. This is indicative of a large number of individual entities, all capable of under going a low

probability spontaneous event. The event then results in the individual entity being taken out of the system, as typified by the emission decay of radioactive isotopes. The decrease in infectious centre titre observed with the 2μg/ml and 0.2μg/ml 4'-s-EtdU treatments, corresponded to a half-life of approximately 4 and 6 days, respectively. The difference between the 2 values was presumably due to the lower concentration of 4'-s-EtdU having a decreased inhibitory effect on cellular DNA synthesis.

3.3.3 **The effect of 4'-s-EtdU on viral persistence and latency in NS0 cells**

The suppressed levels of virus reactivation, was not due to a reduction in the number of cells infected with MHV-68. There was no significant difference in the number of cloned cell lines that harboured virus DNA, generated from either the persistently infected NS0 cell cultures 21 days after treatment with 2μg/ml 4'-s-EtdU, or the untreated infected cell cultures, cloned at the same time. The proportion of clones derived from the untreated culture, that were MHV-68 DNA PCR positive, was higher (15/23) than with the treated culture (11/20). However, the ratios were sufficiently similar, to give an acceptable probability (P = 0.23) that the difference occurred though chance (i.e. getting only 11 positives from 20 randomly selected individuals, each with an individual probability of 15/23 of being positive).

Since the exact proportion of virus positive cells in either the treated or untreated cultures was unknown, it is possible, by application of the binomial theorem, to determine the relative probability of achieving the PCR results for all possible ratios of virus positive cells (*see figure 3.2.7*). The area of overlap between the two curves accounts for approximately 60 to 70% of the total area under either of the two curves and approximately 47% of the total combined area. The area under the curves representing all possible proportions of cells harbouring virus, within the cultures as a whole. This further confirms that the ratio of virus positive cells, within the treated and untreated cultures, was unlikely to be of significant difference.

Virus could reactivated from all 4 of the virus genome positive NS0 clones derived from the 2μg/ml 4'-s-EtdU treated cultures, that were retrieved from liquid nitrogen storage, as determined by infectious centre assay. The frequency of infectious centres for all 4 cells lines was approximately $1/10^5$ cells. These results indicate that the MHV-68 DNA harbouring cells, in the 4'-s-EtdU treated cultures, contained viable virus genomes but spontaneous reactivation events occurred at a very low efficiency. All the clones will have under gone over 20 rounds of cell division in the absence of 4'-s-EtdU during the cloning process alone. Despite this, the number of cell undergoing reactivation was lower than the average number from the parental culture, which at the point of cloning was $1/10^3$ cells. This implies that the cell lines were derived from infected cells in the parental culture, which were not prone to reactivation or 'tightly' latent. Therefore the implication is that it would be impossible to completely eliminate cells capable of reactivation, since even cells which are 'tightly' latent, will reactivate at a low level.

3.3.4 **The effect of 4'-s-EtdU on viral persistence in NS0 cells after treatment withdrawal**

On withdrawal of treatment after 21 days, there were marked differences between the cultures treated with 0.2μg/ml 4'-s-EtdU and those treated with 2μg/ml 4'-s-EtdU. The cell associated infectious virus titres in the cultures treated with 0.2μg/ml 4'-s-EtdU, remained at the withdrawal level, between 100 to 200 pfu per 10^6 cells, for the first week then slowly increased until reaching the original pre-treatment level by 18 days post withdrawal. Likewise, the infectious centre titre slowly increased reaching comparable levels to the pre-treatment cultures by approximately 2 weeks post withdrawal.

Neither the cell associated infectious virus titres or the infectious centre titres of the 2μg/ml 4'-s-EtdU treated cultures returned to the original pre-treatment level. Cell associated infectious virus became detectable from the first time point after withdrawal of 4'-s-EtdU. However, the levels remained stable over the latter 3

weeks of the experiment, being between 100 to 500 times lower than was observed with the untreated culture. This supports the argument that the number of latently infected cells that would readily reactivate had been permanently reduced by the presence of the 4'-s-EtdU. Likewise, the infectious centres also remained at a relatively suppressed level, comparable with the observed titres at the point of 4'-s-EtdU withdrawal. The fact that the cultures treated with 0.2μg/ml 4'-s-EtdU did return to the pre-treatment levels is hard to rectify. However, the fact that the lower dose 4'-s-EtdU treatment at the time of withdrawal, had not yet achieved maximal inhibition of the productive replication of the virus, may have had a bearing on the final outcome, within the persistently infected culture.

3.3.5 **The effect of 4'-s-EtdU on cell lines that do not normally harbour persistent virus**

Little is known about the effects of anti-virals on gammaherpesvirus infections of cell lines that do not normally support viral persistence. This is because the most commonly studied gammaherpesvirus, EBV does not readily undergo productive replication *in vitro* and then only in BL and LCL cell lines. However, productive viral replication plays an important role in any virus infection, with respect to transmission and often disease.

Infection of both BHK and MGC7 cells induces cell death. Neither cell line could be protected for any length of time with 0.2μg/ml 4'-s-EtdU. The MGC7 cell cultures went into crisis after approximately 2 to 3 weeks post infection. Similar results were achieved using BHK cells infected at 0.04 pfu per cell, where the cultures died after six to 7 days. This was presumably due to an incomplete inhibition of productive viral replication. 0.2μg/ml inhibits plaque formation in BHK cells over a 4 day period, but could not entirely eliminate infectious virus from NS0 cell lines, which are predominately latently infected.

At a concentration of 2μg/ml, 4'-s-EtdU protected MGC7 cultures from undergoing

total crisis after being infected at a MOI of 1. Once established, the MHV-68 infected MGC7 cells could be grown in the presents of 2μg/ml 4'-s-EtdU for over 2 months without any obvious cytopathic effects. The virus was however not eliminated since on withdrawal of the treatment after 2 months, productive virus replication re-initiated, killing every cell in the culture. No virus antigens were detectable in the 4'-s-EtdU treated controls, by fluorescent immunostaining at this time. Limiting dilution analysis carried out at 32 days post infection, in the presence of 2μg/ml 4'-s-EtdU, revealed that MHV-68 could be reactivated from 1/3 of the clonal cell lines, after 7 days 4'-s-EtdU withdrawal. This clearly showed that at least 1/3 of the cells in the culture were still infected. These results further suggest that successful productive replication had been entirely inhibited. If limited productive replication had occurred within the culture, then one might expect the number of uninfected cells to be far lower since all proved infectable on withdrawal of treatment and 4'-s-EtdU does not prevent the establishment of infection. However, some viral gene expression was likely to have occurred in the infected cells, since viral TK is believed necessary for the inhibitory properties of 4'-s-EtdU.

3.3.6 **Viral persistence in 4'-s-EtdU treated MGC7 cells**

The cultures were originally infected, in suspension, at a MOI of 1. On the assumption that the infection of cells is a random process, determined by random collision events, then using the Poisson distribution it is to be expected that 63% of the cells would become infected. Being able to reactivate virus from only 4/12 cloned cultures, shows a significant decrease in the number of infected cells present in the cultured, compared to the predicted number ($P = 0.036$ using the binomial analysis, assuming each had a 63% probability of being virus positive). Clearly this analysis assumed that all infected cells were able to reactivate, however it was considered a reasonable assumption. Based on the reactivation studies carried out by myself and others (Dr L. Terry personal communication), the time of 4'-s-EtdU treatment had no obvious effect on the virus harboured in MGC7 cells, with respect to reactivation efficiency, as was the case for the persistently infected NS0 cell lines.

The decrease in the number of infected cells was probably due to the cell death that occurred after initial infection. The cell death occurred in the presence of 2μg/ml 4'-s-EtdU and resulted in approximately 40% to 50% of the cells dying. On the assumption that only the infected cells died, presumably from either toxic build up of virus specific products or, as is quite likely, high level expression of virus encoded TK, inducing activated 4'-s-EtdU cellular toxicity, then the number of infected cells would be reduced to approximately 13% to 23% of the original cells. Of the original cells, 37% should have remained uninfected, so giving rise to the persistently infected culture in which the infected cells comprise only approximately 26% to 38%.

The fact that MHV-68 was not eliminated from the cultures, implies that the virus can maintain it's genome in rapidly dividing cells that would normally be the target for productive replication in the apparent absence of productive virus replication. The culture doubling time for MGC7 cells is under 24 hours (Dr L. Terry personal communication) and therefore after 32 days of culture all the cells should have divided over 32 times. If the virus was incapable of replication the dilution effect after this time would have been approximately 1 in $4.3x10^9$, equating to there being only 1 infected cell in every $4.3x10^9$. In the absence of closer scrutiny, it is impossible to determine the exact mechanism by which the viral genome maintains itself, however a number of possible scenarios can be speculated upon. Upon infection herpesvirus genomes circularise. The virus can then undergo productive replication and synthesis of linear concatemeric daughter strands or establish latency. HSV-1 that has the VP16 gene made dysfunctional and hence can not undergo productive replication, can infect cells, however, the genome becomes gradually diluted over successive rounds of cell replication (Jamieson *et al*, 1995 and Dr C M Preston *personal communication*). This is presumably due to the fact that HSV establishes latency in neurones and so lacks an Ori P. MHV-68 as with other gammaherpesviruses establishes latency in cells that can replicate and hence will code for an Ori P. In this manner, the circularised replicative intermediate could be replicated by the cell, during S phase of cell division. The replicative intermediate

however, is continuously attempting to undergo productive replication and so early genes, such as TK are expressed, which activates cellular 4'-s-EtdU which in turn, prevents the synthesis of linear daughter strands and hence late gene expression.

An alternative, although similar mechanism, might be that, as with the persistently infected NS0 cells, the cells can potentially support both latent and productive virus replication. However, unlike the persistently infected NS0 cells, the balance in MGC7 cells is heavily in favour of productive and hence lytic replication. The 4'-s-EtdU prevents the lytic replication from taking place and so the culture establishes a highly unstable form of latency. Attempts to reactivate cause the sporadic production of TK which activates 4'-s-EtdU and prevents it from happening. There is evidence from HSV research that ACV can cause HSV-1 to go latent in primary neuronal cultures, *in vitro*, which would otherwise be productively infected (Wilcox *et al*, 1988 and 1990). A third possible mechanism might involve the synthesis of sufficient linear genomes to allow the re-circularisation of virus genomes and so prevent the virus from becoming diluted out. However, if this was occurring one might expect these daughter strands to be packaged into virions and so go on to infect other cells. 4'-s-EtdU does not prevent the establishment of infection in either BHK, MGC7 or NS0 cells. In this manner over a period of time, one might expect the culture to either go into crisis, as was the case with the 0.2μg/ml 4'-s-EtdU treatment, or the number of infected cells to gradually increase. However, neither of these phenomena were observed.

3.3.7 **The of effect of MOI on the ability of 4'-s-EtdU to protect BHK cells from MHV-68 infection**

The anti-viral effects of 4'-s-EtdU was less able to protect BHK cells from the MHV-68 induced cytopathic effects, than it was MGC7 cells. 4'-s-EtdU resistant virus mutants also arose far more readily from infected BHK cells than with either infected NS0 or MGC7 cells. 4'-s-EtdU at 2μg/ml protected the BHK cell cultures at a MOI of 0.04 but did not protect at a MOI of 4 and only partially protected at an

MOI of 0.4. This was presumably due to the fact that MHV-68 infection is more cytotoxic in BHK cells than in MGC7 cells. MGC7 cells have constitutive expression of the SV40 large T antigen at the permissive temperature. Expression of large T antigen is known to bind tumour suppresser genes such as P53 and Rb and has the effect of driving cells into continuous proliferation and inhibiting apoptosis (Schaffhausen 1982 and Mole *et al*, 1987). This would probably make the cells more tolerant to the toxicity virus encoded products, as well as activated 4'-s-EtdU. Expression of large T antigen could therefore account for the fact that 4'-s-EtdU protects MGC7 cells but not BHK cells, at high MOI, and that MHV-68 takes 6 days to produce a plaque in MGC7 cells and only 4 days with BHK. However, it is also possible that MHV-68 replicates faster in BHK cells, since BHK cells may provide a more conducive environment for productive viral replication than the MGC7 cells.

Despite not being able to protect BHK cells at high MOI, 4'-s-EtdU at 2μg/ml could protect BHK cells at low MOI. The virus was not lost from these cultures for at least 10 days of continual treatment. The doubling time of BHK cells is approximately 24 hours therefore the dilution effect in the absence of viral replication would have been over 1000 fold. The numbers of infected cells, however was not quantified to determine whether the levels had decreased or not. Studies could not be carried out much further than 2 weeks since the infected BHK cell cultures were highly prone to the spontaneous generation and outgrowth of 4'-s-EtdU resistant virus mutants.

3.3.8 **The spontaneous generation of 4'-s-EtdU resistant virus**

No evidence for the spontaneous generation 4'-s-EtdU resistant virus mutants was found with the long-term 4'-s-EtdU treated persistently infected NS0 cell cultures, at either 0.2 or 2μg/ml. This was perhaps surprising since 4'-s-EtdU treatment at 0.2μg/ml allowed low level chronic replication of the virus within the culture. However, NS0 cells support predominately a latent infection and so the level of productive virus replication, as compared to either BHK or MGC7 cells, was very low. 4'-s-EtdU resistant mutants spontaneously arose from persistently infected

MGC7 cells only after 10 weeks of 4'-s-EtdU treatment at 2μg/ml. The fact that 4'-s-EtdU resistant mutants arose from BHK cells far more readily than from either NS0 or MGC7 cells could be due to the cellular environment being more conducive to productive replication.

3.3.9 **4'-s-EtdU resistant MHV-68 isolates**

The 4'-s-EtdU resistant mutants that spontaneously arose from the persistently infected MGC7 cells were cloned and partially characterised. Six resistant isolates, MERV 1 to 6, were cloned by 3 rounds of limiting dilution plaque purification. 4'-s-EtdU at concentration up to 10μg/ml had no effect on the plaque forming ability of any of the resistant virus clones, however all remained sensitive to the anti-viral effects of ACV. Since 4'-s-EtdU has a similar viral range similar to ACV, being effective at inhibiting the productive replication of HSV and VZV but not HCMV (*see Table 3.1*), it was assumed to be activated and incorporated in a similar manner.

ACV resistant HSV-1 mutants can be isolated from immunosuppressed, transplant patients or AIDS patients after long-term treatment with ACV. Similarly, mutants can be generated *in vitro*, by propagating polyclonal virus stocks, in the presence of ACV and selecting for resistant virus. The most common mutations responsible for conferring ACV resistance, result in the functional deficiency of the viral encoded TK (Smith *et al*, 1980, Martin *et al*, 1985 and Hill *et al*, 1991). This is commonly caused by the insertion or deletion of a single nucleotide resulting in a frame shift mutation and so resulting in the complete loss of viral TK activity (Sasadeusz *et al*, 1997). Although TK deficient mutants replicate efficiently *in vitro*, they appear greatly attenuated *in vivo* (Efstathiou *et al*, 1989 and Coen *et al*, 1989). Less common mutations that result in ACV resistance, are caused by base pair substitution in either the TK encoding gene (Darby *et al*, 1981, Martin *et al*, 1985 and Hill *et al*, 1991) or the viral polymerase encoding gene (Field *et al*, 1980 and Collins *et al*, 1989). Base pair substitutions can give rise to TK alteration mutations, where amino acid alterations in the viral TK, change the substrate specificity,

resulting in the failure to recognise and phosphorylate ACV. Similarly amino acid alterations in the viral DNA polymerase can result in failure to recognise ACV triphosphate as a nucleotide so fails to incorporate the anti-viral into the viral DNA during synthesis. Mutant polymerase strains of HSV often have reduced sensitivity to the anti-viral pyrophosphate analogues, such as foscarnet (PFA) and phosphonoacetic acid (PAA) (Morfin *et al*, 1996).

The fact that the MERV clones remained sensitive to ACV suggests that they are not TK deficient mutants, since TK is required for ACV sensitivity. Mutations that alter the ability of HSV TK to convert a specific nucleoside analogues to the monophosphate derivative, tend to confer resistance to most other TK-dependant anti-virals. However, only the pyrimidine analogues, such as BVdU and BVaraU, require virus encoded TK to convert the nucleoside monophosphate to the diphosphate form. Mutations that affect the TK conversion of nucleoside monophosphates to the diphosphate form generally do not affect the conversion of nucleosides to their monophosphate forms. The selective resistance of the MERV clones therefore suggests that resistance was achieved via TK mutations that specifically affect the phosphorylation of 4'-s-EtdU monophosphate to 4'-s-EtdU diphosphate. Alterations to the MHV-68 TK, which affect the conversion of 4'-s-EtdU to 4'-s-EtdU monophosphate, or to the viral polymerase are less likely. Studies carried out on HSV resistance to anti-viral pyrimidine analogues such as BVdU and BVaraU have shown that selection with either BVdU or BVaraU alone did not give rise to mutants that lost sensitivity PAA and PFA. To achieve this, BVdU or BVaraU had to be combined with either pyrophosphate analogues or ACV (Morfin *et al*, 1996). However, neither TK or polymerase mutations can be ruled out since MHV-68 is not HSV and BVdU / PAA double resistant HSV mutants, can remain sensitive to ACV. To determine the exact mechanism by which the 4'-s-EtdU resistance was acquired, both the MERV TK encoding gene (ORF 21) and the viral polymerase gene (ORF 9) would have to be genetically sequenced.

Chapter 4: The effect of 4'-s-EtdU treatment on the pathology of the MHV-68 infection of BALB/c mice.

4.1.1 Summary

Experiments were carried out to determine the long-term effect of 4'-s-EtdU treatment from day 3 post infection and the effect of prophylactic 4'-s-EtdU treatment on the pathology of the MHV-68 infection of BALB/c mice 3 to 4 weeks of age. Treatment from day 3 post infection, significantly shortened the acute lung infection and had a pronounced inhibitory effect on viral persistence in the lung. However, the treatment failed to prevent both the post acute splenomegaly and the establishment (and maintenance) of viral latency in splenocytes. Prophylactic 4'-s-EtdU treatment, prevented any detectable productive viral replication in the lungs of mice infected via the intra nasal route. Furthermore, the mice failed to sera convert to late viral antigens, undergo splenomegaly or establishment of viral latency in splenocytes. However, the treatment did not prevent the establishment of a persistent infection in the lung tissue, which was maintained up to day 54 post infection, in the apparent absence of both productive viral replication and latently infected circulating B-cells. On withdrawal, the mice sera converted to late viral antigens and the virus was shown to seed to the spleen. Surprisingly, prophylactic 4'-s-EtdU treatment on mice infected via the intra peritoneal route, also resulted in mice failing to develop splenomegaly and half the mice failing to establish splenic latency. The lungs were not the predominant site for viral persistence outside lymphoid organs in the 4'-s-EtdU treated mice, infected via the intra-peritoneal route, pointing to a third site of viral persistence. The study clearly demonstrates the importance of productive viral replication in the dissemination from the site of acute infection to other cellular compartments. The study also provides clear evidence for both chronic and latent viral persistence in the lungs of mice infected via the intra-nasal route. The evidence highlights the potential importance of the site of primary infection, not only with respect for virus transmission, but on the gross pathology of gammaherpesvirus infections

4.1.2 Advances in the clinical use of nucleoside analogues to combat herpesvirus infections

Over the previous 40 years, there have been major advances in the development of anti-herpesvirus compounds. The first anti-herpesvirus agents used clinically were IdU, TFT and vidaravine. All 3 had significant toxicity and have been largely superseded by ACV. ACV is specifically activated by herpesvirus encoded TK to the active form and hence has very low toxicity. Despite the low level of adverse side effects associated with AVC treatment, ACV has a low solubility, low bioavailability and a short intracellular half-life. Recently a new generation of highly soluble anti-viral pro-drugs which have greatly increased bioavailibility but equally low toxicity. Valiciclovir (VACV) is a L-valyl ester derivative of ACV. VACV is highly soluble and converted to AVC by first pass metabolism in the liver (Demiranda *et al*, 1994 and Burnette *et al*, 1994). The conversion of VACV to ACV is carried out by an atypical esterase, termed valaciclovir hydrolase (Burnette *et al*, 1995). The oral bioavailability of ACV following oral administration of VACV is 54% (Murray *et al*, 1995 and Soullawton *et al*, 1995). This compares with only 12 to 20% for oral administration of ACV (Acosta *et al*, 1997). VACV has proven effective in the treatment of both clinical HSV I, HSV II and VZV infections (Patel *et al*, and 1997, Fife *et al*, 1997, Bodsworth *et al*, 1997, Marley *et al*, 1997 and Tyring *et al*, 1998). VACV also has been shown to be effective at preventing CMV recurrent infections in immunocompromised patients (Feinberg *et al*, 1998 & Griffiths *et al*, 1998). However, recurrence of HSV replication has been observed following cessation of VACV treatment in animal models (Field *et al*, 1995a & 1995b, Thackray *et al*, 1996a & b & 1997).

Famcyclovir (FCV) is a diacetyl 6-deoxy derivative of PCV. As with ACV, PCV is an acyclic guanosine analogue specifically activated by herpesvirus TK. The oral

bioavailabilty of PCV following oral administration of FCV is 77% and similarly to VACV is converted, to PCV, by first pass metabolism in the liver (Gill *et al*, 1996, Crumpacker *et al*, 1996, Luber *et al*, 1996 and Borg *et al*, 1997). The conversion of FCV to PCV involves di-deacetylation to 6 deoxcy PCV and then 6' oxydation to PCV by the liver molyddemum hydroxylase, aldehyde oxydase (Clarke *et al*, 1995 and Rashidi *et al*, 1997). Oral FCV is affective in the treatment of HSV I, HSV II and VZV recurrent infections (Sack *et al*, 1996, Tyring, *et al*, 1995 & 1996, Mertz *et al*, 1997 and Schacker *et al*, 1998). FCV appears to be able to suppress the establishment of latency by HSV (Thackray *et al*, 1996a & b & 1997 and Field *et al*, 1997) and recurrence of HSV replication following cessation of treatment has not been observed in animal models (Field *et al*, 1995a & b, Thackray *et al*, 1996a & b & 1997). Oral FCV as well as topical PCV, IdU and PFA have proven effective in the treatment of HSV labalis (Spruance *et al*, 1997a and b and Bernstein *et al*, 1997). FCV has also been shown to be and effective treatment of hepatitis B virus infections (Piqueras *et al*, 1997 and Lau 1998) due to the ability of PCV-TP to inhibit hepadnavirus reverse transcriptase (Zoulin *et al*, 1995 and Dannaoui *et al*, 1997).

4.1.3 **The clinical use of anti-virals that are virus non-exclusively virus activated**

Betaherpes viruses do not encode a homologue to the HSV I TK gene and therefore ACV and PCV have proven ineffective at treating CMV disease in immunocompromised patients. The main treatment for which, along with foscarnet, has been a further acyclic guanosine analogue, GCV (Drew *et al*, 1995 and Balfour *et al*, 1996). GCV has a higher level of toxicity than either ACV, PCV or their pro-drug derivatives (Thust *et al*, 1996). As with ACV and PCV, GCV has a low bioavailability, which has led to the development of an oral GCV pro-drug similar to FCV (Krasny *et al*, 1995). The monophosphate nucleotide analogue HPMPC has also proved highly effective against ocular CMV disease, as well as the treatment of ACV resistant HSV labelis in AIDS patients. HPMPC has a very long intracellular half life and can inhibit a wide variety of herpesviruses (Alrabiah *et al*, 1996 and Freeman *et*

al, 1996).

The non-virus activated nucleoside analogues in clinical use are a relatively biochemically diverse group. The dideoxynucleosides, such as didanosine (ddI) and dideoxycytodine (ddC), and the dideoxynucleoside derivatives, such as zidovudine (AZT), stavadin (d4T) and 2'-deoxy-3'-thiacytidine (3CT), are all obligate chain terminators (St. Clair *et al*, 1972 and 1991). All are used in the treatment of HIV infection and AIDS, and have a range of side effects that include bone marrow toxicity and neuropathy. HPMPC is a nucleotide mono-phosphate analogue and hence does not require activation by viral kinases. HPMPC has anti-viral activity against a broad range of herpesviruses and papillomaviruses. HPMPC has a very long intracellular half life and is used in the treatment of ACV resistant HSV-2 and HCMV retinitis in AIDS patients (Cundy *et al*, 1995, Lalezari *et al*, 1995 and Polis *et al*, 1995). The main side effect of HPMPC is nephrotoxicity. Ribavirin is a ribonucleoside analogue and has anti-viral properties against a variety of RNA viruses (Eriksson *et al*, 1977 and Toltzis *et al*, 1988). Ribavirin is used in the treatment of respiratory syncytial virus infections, Lassa fever and Hantaan virus haemorrhagic fever (Hall *et al*, 1983, Andrei *et al*, 1993). Ribavirin is both mutagenic and has side effects that include conjunctivitis, reticulocytosis and bone marrow suppression.

4.1.4 **The role of productive replication in EBV pathology**

Productive virus replication plays a critical role in the transmission of virtually all viruses. It is also commonly associated with viral disease pathology. Transmission of EBV is generally achieved by the shedding of infectious virions in saliva. The source of which, and probably the site of initial infection, are the epithelial cells of the oro-pharynx. Productively infected epithelial cells have been observed in association with (Lemon *et al*, 1977). The role of the epithelial cell infection *in vivo*, is contentious since the treatment of IM patients with ACV has proved ineffective and B-lymphocytes can also support limited productive replication *in vivo* (Pang *et*

al, 1997 and Niedobitek *et al*, 1997).

The only disease directly associated with productive replication of EBV in the epithelial cells of the oro-pharynx, is known as hairy oral leukoplakia (HOL). HOL is a complication associated with AIDS patients and can be successfully treated with anti-virals such as ACV and ACV derivatives (Resnick *et al*, 1988 and Greenspan *et al*, 1990). Productive replication in the oro-pharynx may also play an important role in IM since EBV transformation of B-lymphocytes, both *in vitro* and *in vivo*, requires the addition of exogenous virus or the reactivation of latent virus (Pope *et al*, 1968, Rickenson *et al*, 1986, Morgan *et al*, 1988 and Dosch *et al*, 1991). It has also been observed that the prevalence of EBV associated post-transplantation LPD is far higher in children who are EBV sera-negative prior to immunosuppression, and then become primarily infected (Cox *et al*, 1995 and Newell *et al*, 1996). The deciding factor in whether a post-pubescent develops IM after primary infection, may well depend on the number of B-lymphocytes initially infected. This is going to depend on 2 factors; the titre of the initial infecting dose or the severity of the acute infection of the oro-pharynx.

4.1.5 **The MHV-68 *in vivo* gammaherpesvirus model for anti-viral testing**

It is difficult to determine the potential therapeutic benefits of nucleoside analogues in the treatment of EBV primary infection, because humans are the only natural host for the virus and infection only becomes apparent after the onset of IM symptoms. Although MHV-68 is genetically divergent from EBV, the 2 viruses appear to have similar patterns of infection and cellular tropism, in their respective natural hosts. MHV-68 also has similar anti-viral susceptibilities to EBV and so makes a good model for the study of potential therapeutic treatments, under controlled experimental conditions (for review see Sunil-Chandra 1994b). Previous studies have shown that ACV taken orally from day 3 post intra-nasal infection, significantly reduced the virus titres in the lung during acute infection. However, the treatment failed to shorten the length of time of the acute infection or greatly affect the shape

of the lung infectious virus titre profile. From the anti-virals previously tested on MHV-68 *in vivo*, only cidofovir has been shown to effectively combat virus replication in infected mice (Neyts *et al*, 1998). Anti-viral treatment of MHV-68 has also been shown to be ineffective at preventing the establishment of virus latency in splenocytes, although the appearance of splenic infectious centres was delayed in treated animals (Sunil-Chandra *et al*, 1994c and Barnes *et al*, unpublished data).

4.1.6 **The effect of 4'-s-EtdU treatment on the MHV-68 infection of mice.**

Experiments have been carried out comparing the potency of 4'-s-EtdU to ACV *in vivo*. Treatment of mice with 4'-s-EtdU (1mg/mouse per day) from day 3 post intra-nasal infection resulted in all detectable infectious virus being eliminated from the lungs of the treated mice within 4 days of treatment. The ACV treatment, carried out in parallel at the same dose (1mg/mouse/day), other than slightly reducing the lung virus titres, had little affect on the acute lung infection. This data clearly demonstrated the increased potency of 4'-s-EtdU over ACV *in vivo*. Despite this, the 4'-s-EtdU treatment failed to prevent the establishment of viral latency in splenocytes. As with ACV treatment, 4'-s-EtdU treatment resulted in a significant delay in the appearance of splenic infectious centres, with virus latency levels remaining suppressed up till experimental termination (day 13 post infection). The study did not therefore established whether the 4'-s-EtdU treatment would affect viral latency levels long-term, in the spleen (Barnes *et al*, unpublished data).

4.1.7 The treatment of intra-nasally MHV-68 infected mice, with 4'-s-EtdU from day 3 post infection.

To determine the long-term effects of 4'-s-EtdU treatment on the MHV-68 infection *in vivo*, 61 female BALB/c mice, aged 3 to 4 weeks, were infected with $4x10^5$ pfu MHV-68, via the intra-nasal route. For long-term drug treatment of mice, the 4'-s-EtdU was to be administered via drinking water (containing soluble 4'-s-EtdU). To explore the feasibility of this approach, a further group of 6 mice were infected with $4x10^5$ pfu MHV-68, via the intra-nasal route. The group of 6 infected mice were split into 2 groups of three mice, one of which remained on normal drinking water, the second were put on drinking water containing 0.3mg/ml 4'-s-EtdU. At day 3 post infection, 3 mice from the group of 65 MHV-68 infected mice, were killed and lungs removed, to assess the establishment of the acute lung infection. To assess the effectiveness of 4'-s-EtdU administration, via drinking water, the 3 drinking water control mice, and the 3 mice on 4'-s-EtdU supplemented drinking water, were also killed and lungs removed. Of the remaining 58 infected mice, 40 were administered 0.2 ml of a 0.5% (W/V) 4'-s-EtdU suspension orally, under mild anaesthesia, using a gavage needle. The remaining 18 mice were administered 0.2 ml water, in a similar manner. The oral administration of the 4'-s-EtdU (or water) was repeated once a day, until day 12 post infection.

Infectious virus assays carried out on the lungs of the drinking water control and the 4'-s-EtdU supplemented drinking water mice, clearly showed that administration of 4'-s-EtdU via drinking water was an effective way of inhibiting the replication of MHV-68 (*see figure 4.1.1*). The drinking water control mice had infectious virus titres between $2x10^3$ and $2x10^5$ pfu per lung, where as none of the treated mice had any detectable infectious virus present. On day 12 post infection, half the treated mice were withdrawn from 4'-s-EtdU treatment, the other half carried on treatment via drinking water, supplemented with 4'-s-EtdU at 0.3mg/ml. Three mock-treated

Figure 4.1.1

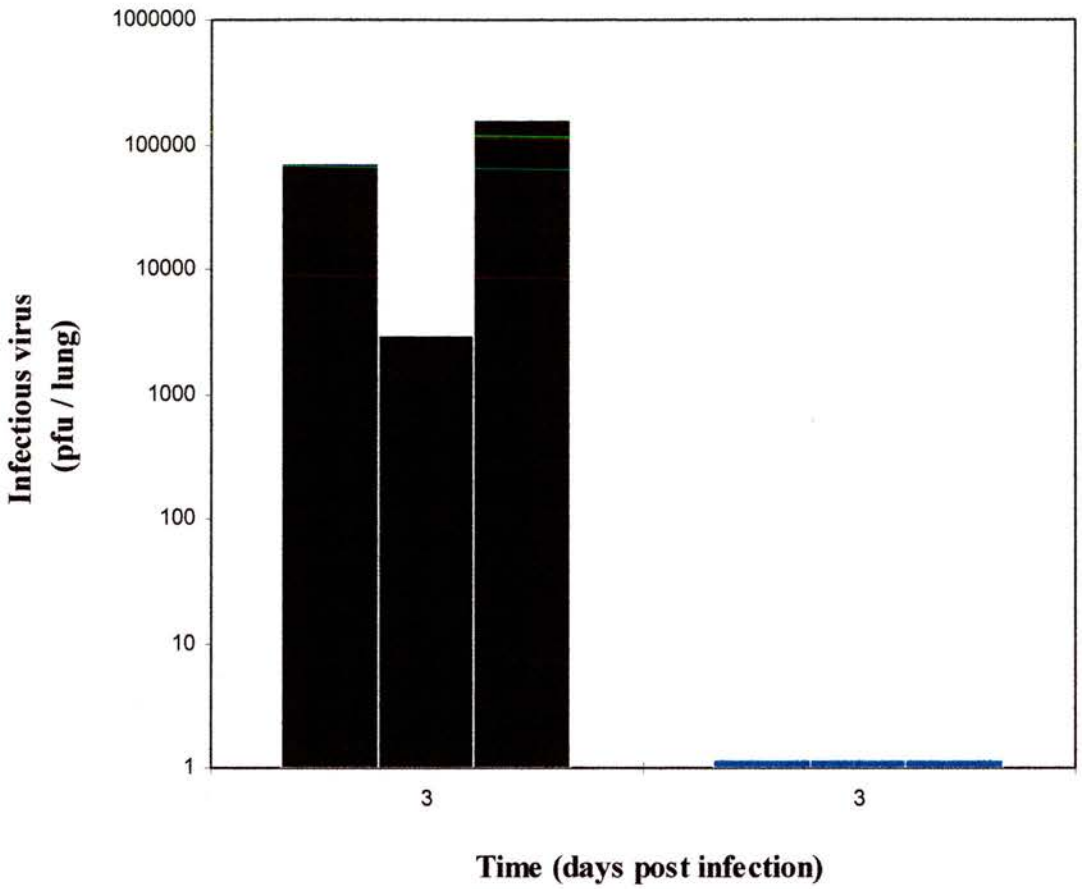


Figure 4.1.1 The infectious virus titres (as measured by direct plaque assay) detected in the lungs of mice infected intra-nasally with $4x10^5$ pfu MHV-68, at day 3 post infection. The mice were either left untreated (black) or treated, via drinking water supplemented with 4'-s-EtdU (0.3mg/ml) from day 0 post infection (blue). Three mice were sampled from both the treated and untreated groups. The assay limit of detection was 10 pfu per lung.

and 4 treated mice were sampled at days 7 and 12 post in b fection. A further 3 mock-treated, 4 continually treated and 4 treatment withdrawn mice were sampled at days 26, 38, 48 and 61 post infection. At each time point lungs and spleens were removed for analysis.

4.1.8 **The effects of 4'-s-EtdU treatment (from day 3 post infection) on the MHV-68 infection of the lung.**

High titres of infectious virus could be detected in the lungs of mice at day 3 post infection. Infectious virus titres remained high in the mock-treated mice up to day 7 post infection. However, by day 7 post infection, the lungs of the treated mice contained no detectable infectious virus, by direct plaque assay. By day 12 post infection, infectious virus was not detected in the lungs of any of the mice, as was the case for all subsequent time points. (*see figure 4.1.2*). Despite the absence of detectable infectious virus in the lungs, virus could be reactivated, by a lung co-cultivation assay, from all the untreated mice (9/9), sampled at the later 3 time points (days 38, 48 and 61 post infection). Virus could also be reactivated from lung tissue of all the mice withdrawn from treatment (10/10), again sampled at the later 3 time points. However, virus could only be reactivated from the lung tissue of 1/12 mice not withdrawn from treatment (*see figure 4.1.3*).

4.1.9 **The effects of 4'-s-EtdU treatment (from day 3 post infection) on MHV-68 induction of splenomegaly**

Splenomegaly occurred in all the groups of infected mice (*see figure 4.1.4*). At day 7 post infection, the number of splenocytes observed in both the treated and untreated mice was between $6x10^7$ and $7x10^7$ leukocytes per spleen. By day 12 post infection, the number of splenocytes observed in both the untreated and treated mice, increased significantly (P= 0.001 and 0.022 respectively, by student T test). The increase was greater in the untreated than in the treated mice ($1.48x10^7$ as opposed to $1.25x10^7$ leukocytes per spleen), although the difference was not significant (P=0.16 by

Figure 4.1.2

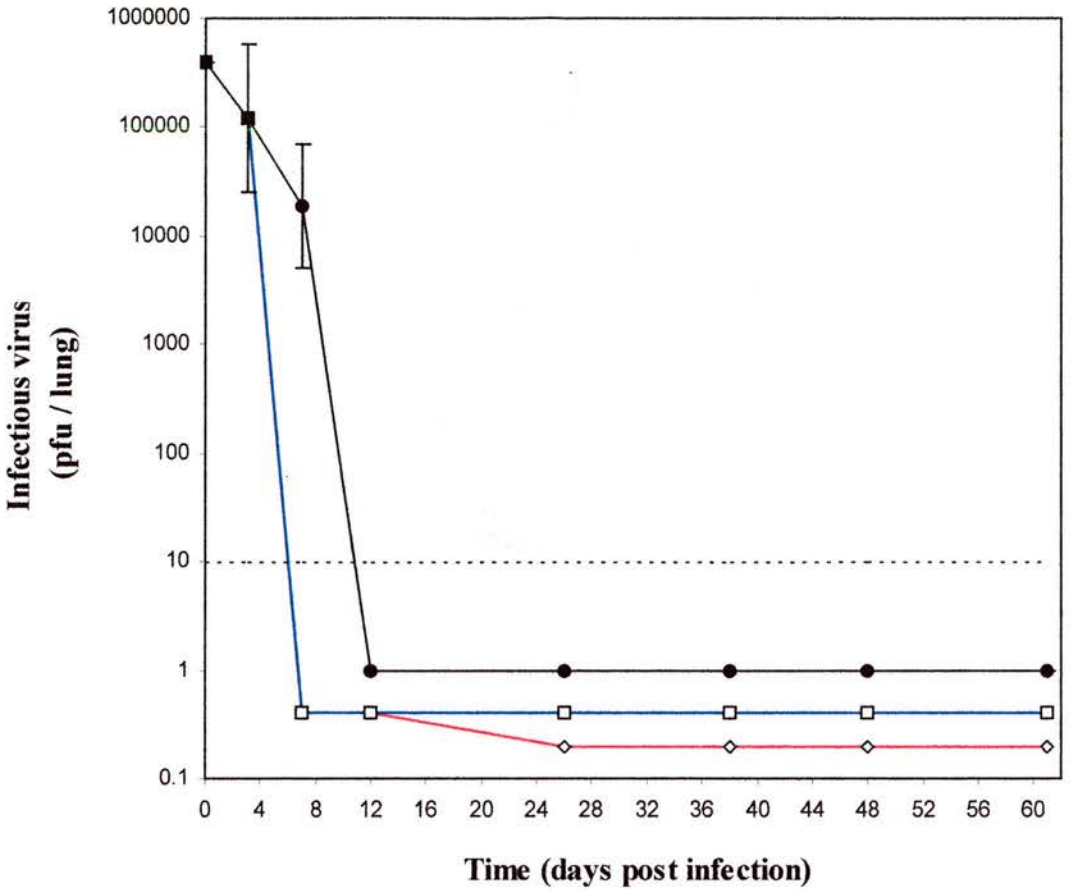


Figure 4.1.2 The infectious virus titres (as measured by direct plaque assay) detected in the lungs of mice infected intra-nasally with $4x10^5$ pfu MHV-68. The mice were either left untreated (black), 4'-s-EtdU treated from day 3 post infection and not withdrawn (blue) or treated from day 3 post infection and withdrawn day 12 post infection (red). Four mice were sampled from the 4'-s-EtdU treated groups at every time point (days 3, 7, 12 26, 38, 48 and 61 post infection). Three mice were sampled from the untreated group at every time point. The values are represented by geometric means and the error bars represent the standard deviation. The dotted line represents the assay limit of detection.

Figure 4.1.3

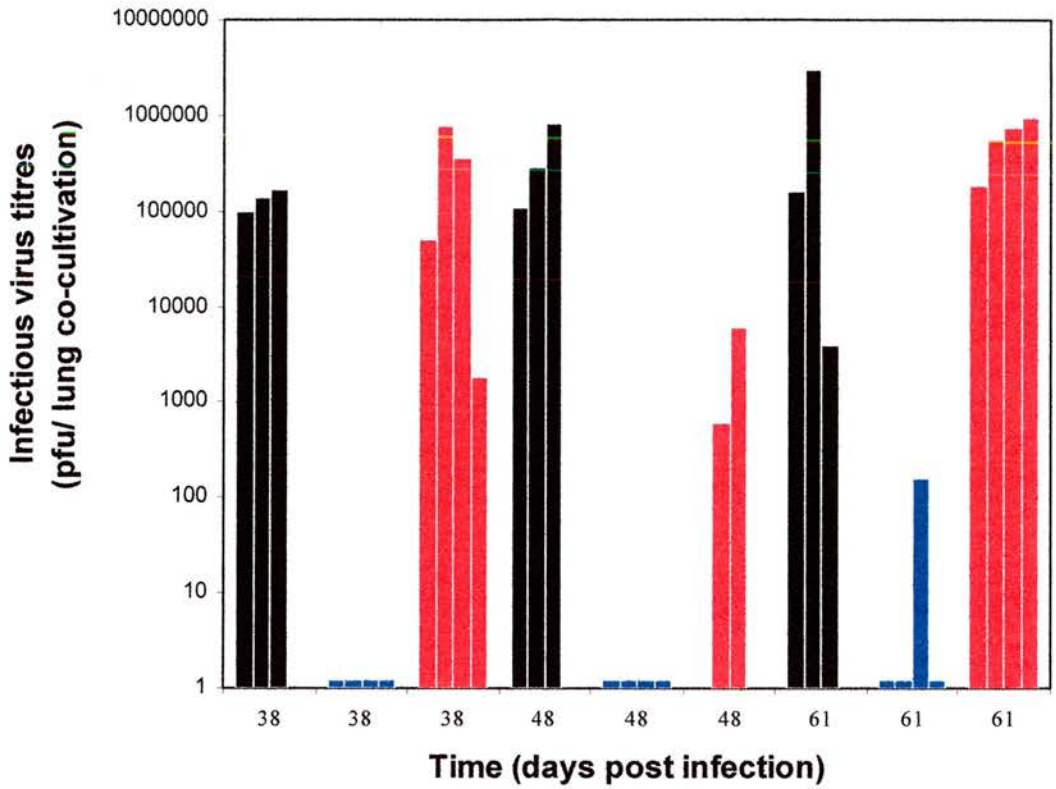


Figure 4.1.3 The levels of infectious virus reactivated (by co-cultivation assay) from the lung tissue of mice infected intra-nasally with $4x10^5$ pfu MHV-68. The mice were either left untreated (black), 4'-s-EtdU treated from day 3 post infection and not withdrawn (blue) or treated from day 3 post infection and withdrawn day 12 post infection (red). Four mice were assayed from the 4'-s-EtdU treated groups at every time point tested (days 38, 48 and 61 post infection), except for day 48 post infection where only 2 of the treatment withdrawn mice were assayed. Three mice were assayed from the untreated group at every time point tested. The assay limit of detection was 10 pfu per lung co-cultivation.

Figure 4.1.4

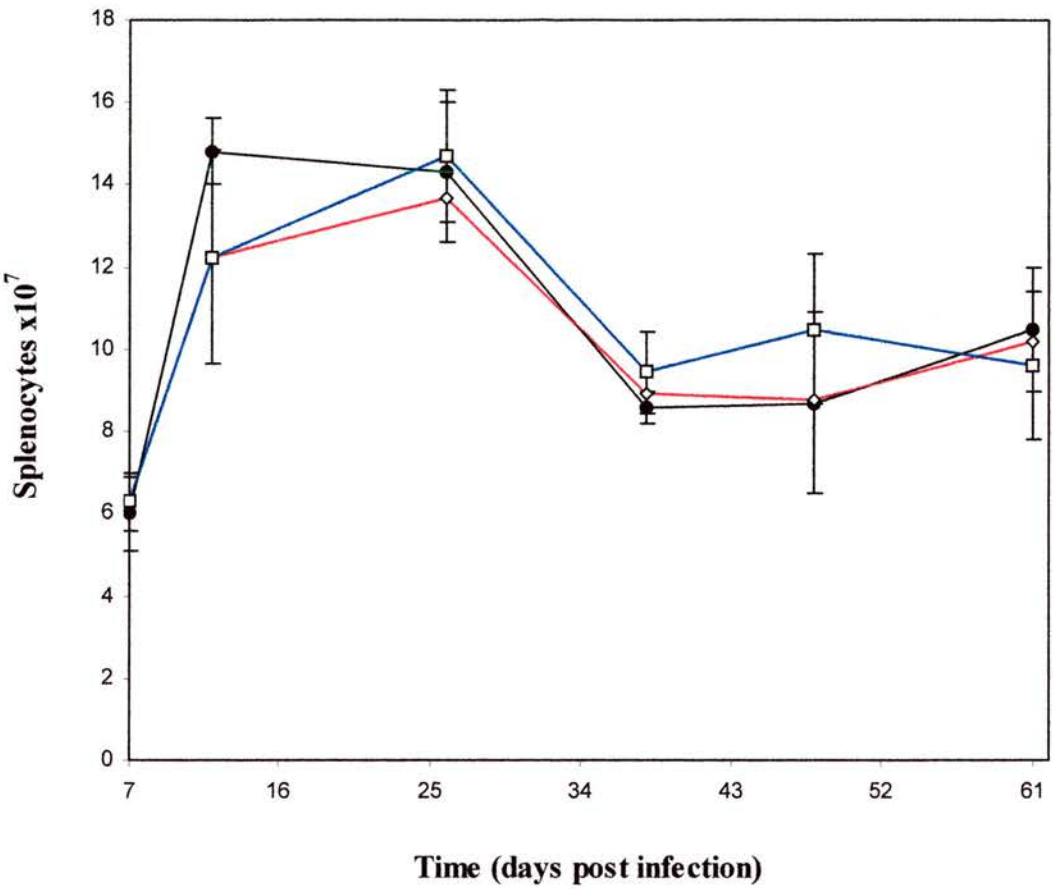


Figure 4.1.4 The number of splenocytes observed in mice infected intra-nasally with $4x10^5$ pfu MHV-68. The mice were either left untreated (black), 4'-s-EtdU treated from day 3 post infection and not withdrawn (blue) or treated from day 3 post infection and withdrawn day 12 post infection (red). Four mice were sampled from the 4'-s-EtdU treated groups at every time point tested (days 7, 12 26, 38, 48 and 61 post infection). Three mice were sampled from the untreated group at every time point tested. The values are represented by arithmetic means and the error bars represent the standard deviation.

student T-test). At day 26 post infection, the number of splenocytes observed in the mock-treated mice and in the mice withdrawn and non-withdrawn from treatment, remained elevated showing no significant differences from the significantly elevated values observed at day 12 post infection (P=0.65, 0.42 and 0.19 respectively, by student T-test). However, by day 38 post infection, the number of splenocytes observed in the mock-treated mice and in the mice withdrawn and non-withdrawn from treatment, had significantly decreased with respect to the numbers observed at the previous time point (P=0.031, 0.0026 and 0.046 respectively, by student T-test). After day 38 post infection, no significant changes in the number of splenocytes was observed in any of the groups of mice, with the numbers remaining significantly lower than observed at day 26 post infection. There was no significant difference in the number of splenocytes observed in any of the groups of mice, at any specific time point.

4.2.1 **The effect of 4'-s-EtdU treatment (from day 3 post infection) on viral latency in the spleen.**

At the first time point tested (day 7 post infection), all the mock-treated mice (3/3) had detectable levels of latently infected splenocytes, as determined by infectious centre assay. By day 12 post infection, the splenic infectious centre titres had peaked, increasing from an average of 7 per spleen to 1767 per spleen (P=0.009 by student T-test). From day 12 post infection, the splenic infectious centre titres decreased, becoming significantly lower (than day 12 post infection) by day 38 post infection. The infectious centre titres remained significantly lower for all subsequent time points (P=0.032, 0.045 and 0.038 respectively, by student T-test). By day 48 post infection, the latent virus titres had reached a base line level, averaging between 5 and 15 infectious centres per spleen (*see figure 4.1.5*).

No latent virus was detectable in the splenocytes of any of the 4'-s-EtdU treated mice at day 7 post infection (0/4), as detected by infectious centre assay. Although infectious centres could be detected in all the treated mice by day 12 post infection,

Figure 4.1.5

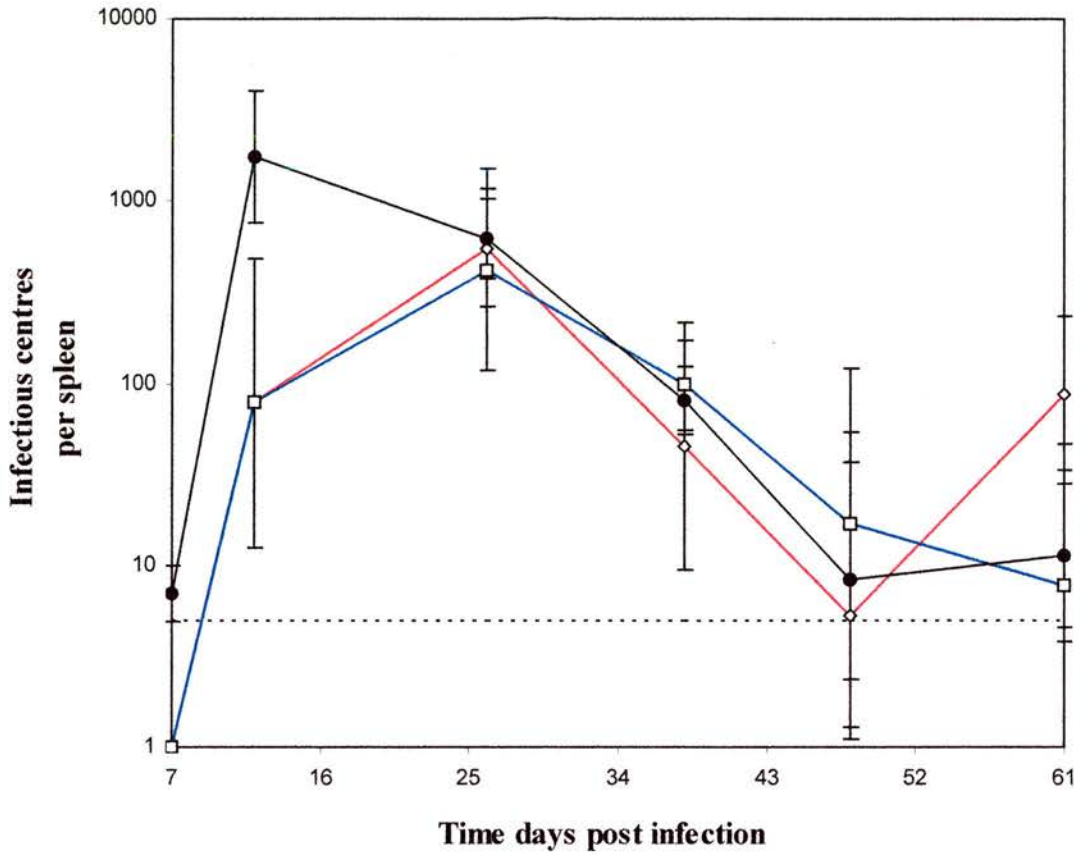


Figure 4.1.5 The latent virus titres (as measured by infectious centre assay) detected in the spleens of mice infected intra-nasally with $4x10^5$ pfu MHV-68. The mice were either left untreated (black), 4'-s-EtdU treated from day 3 post infection and not withdrawn (blue) or treated from day 3 post infection and withdrawn day 12 post infection (red). Four mice were assayed from the 4'-s-EtdU treated groups at every time point tested (days 7, 12 26, 38, 48 and 61 post infection), except on days 26 and 38 post infection where only 3 mice from the treatment withdrawn group were assayed. Three mice were sampled from the untreated group at every time point tested. The values are represented by geometric means and the error bars represent the standard deviation. The dotted line represents the assay limit of detection.

the titres were signifi[illegible] seen in the untreated mice at that time (P=0.047). By day 2[illegible] ll subsequent time points), the infectious centre titres seen in both the mice withdrawn and non-withdrawn from treatment, were indistinguishable from the mock-treated mice.

4.2.2 **The effect of 4'-s-EtdU treatment (from day 3 post infection) on the sensitivity of lung and splenocyte derived MHV-68 isolates**

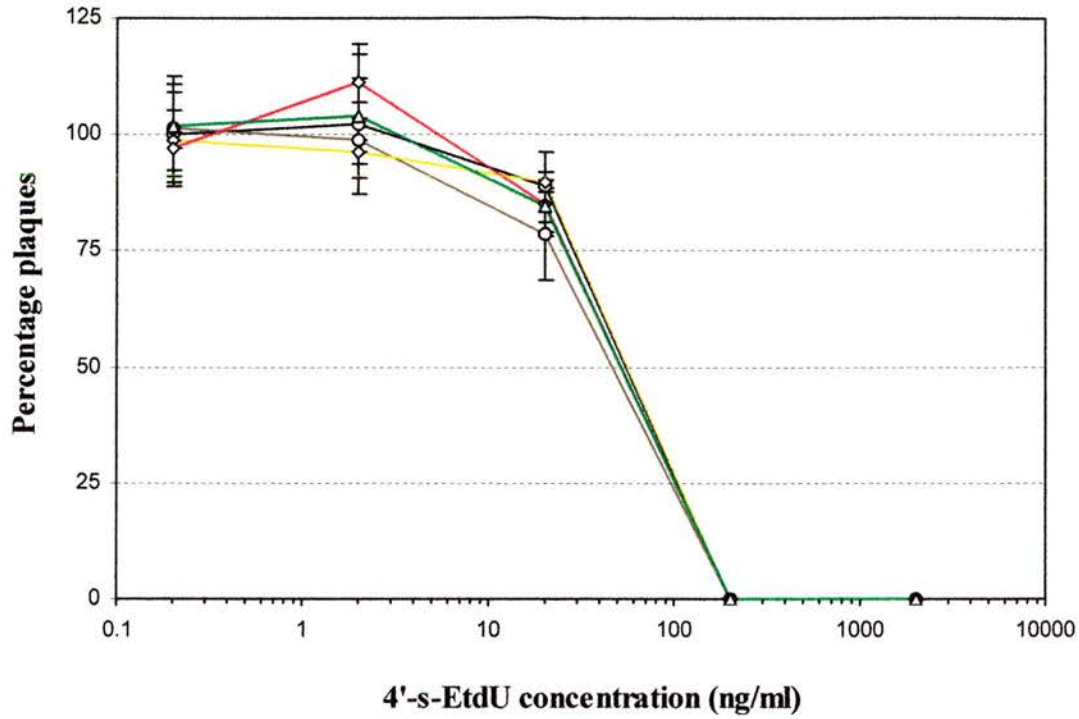
To determine whether the 4'-s-EtdU treatment of infected mice lead to the generation and selection of 4'-s-EtdU resistant virus variants, a number of virus isolates were generated from splenocytes and lung tissues of the infected mice. EC_{50} assays were carried out on virus preparations derived from the lung tissue (by co-cultivation assay) of the mock-treated mice and the mice withdrawn from treatment, sampled at days 38 and 61 post infection (*see figure 4.1.6A & B*). No difference in 4'-s-EtdU sensitivity was observed in the virus reactivated from the lungs of the mice withdrawn from treatment as compared with either the virus reactivated from the lungs of untreated mice or the virus working stock originally used to infect the mice. EC_{50} assays were also carried out on virus preparations derived from splenocytes from the mock-treated mice and in the mice withdrawn and non-withdrawn from treatment, sampled on day 61 post infection (*see figure 4.1.6C, D and E*). Again no difference in 4'-s-EtdU sensitivity was observed between the three groups of mice and the virus working stock used to infect the mice.

4.2.3 **The prophylactic 4'-s-EtdU treatment of intra-nasally infected BALB/c mice**

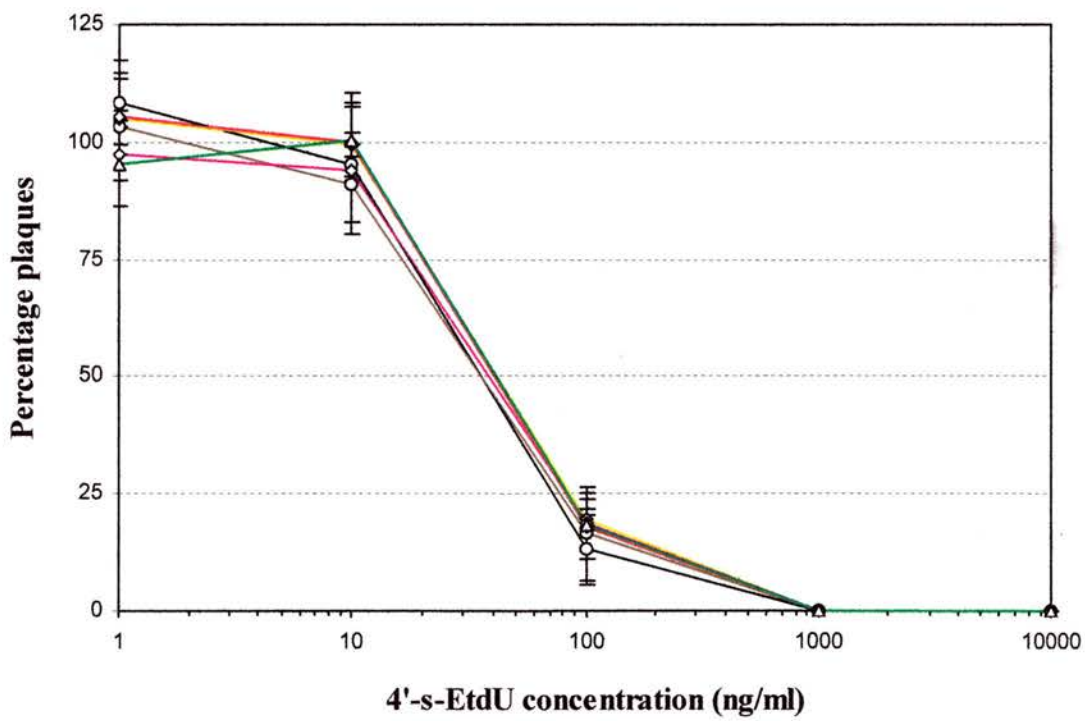
Previous experiments designed to determine the relative importance of the primary acute infection on the establishment of viral latency in B-lymphocytes and general disease pathology, failed to demonstrate a therapeutic benefit in shortening the acute infection. However, anti-viral treatment was initiated only after the acute infection had been allowed to become established. To determine if productive virus

Figure 4.1.6

A



B



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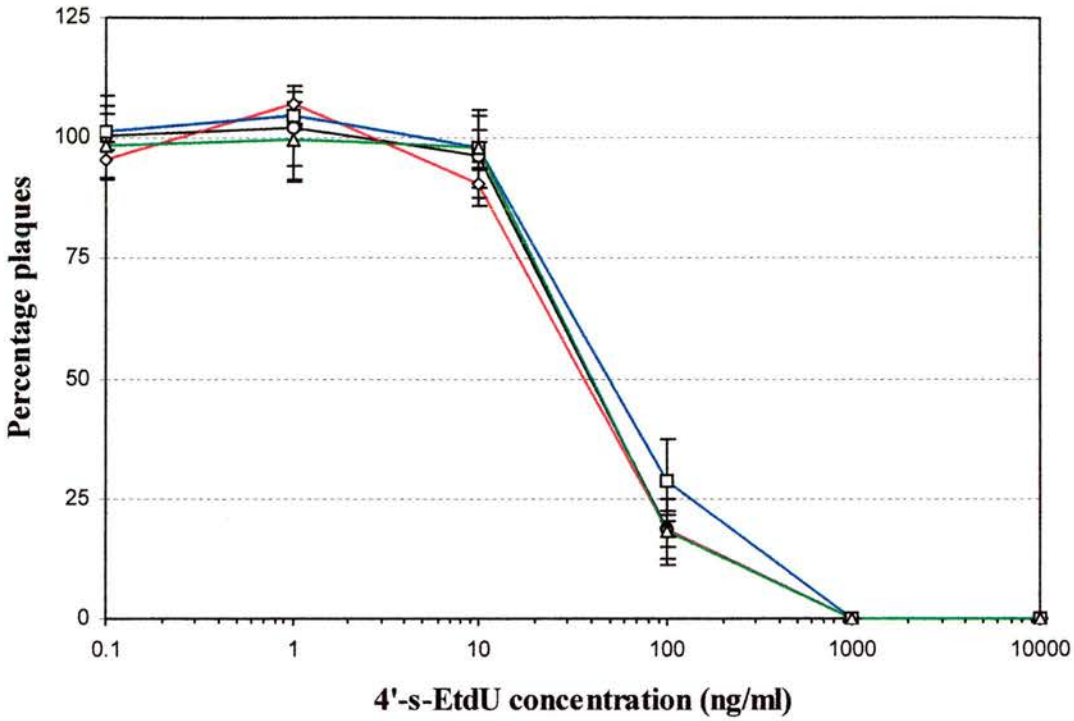
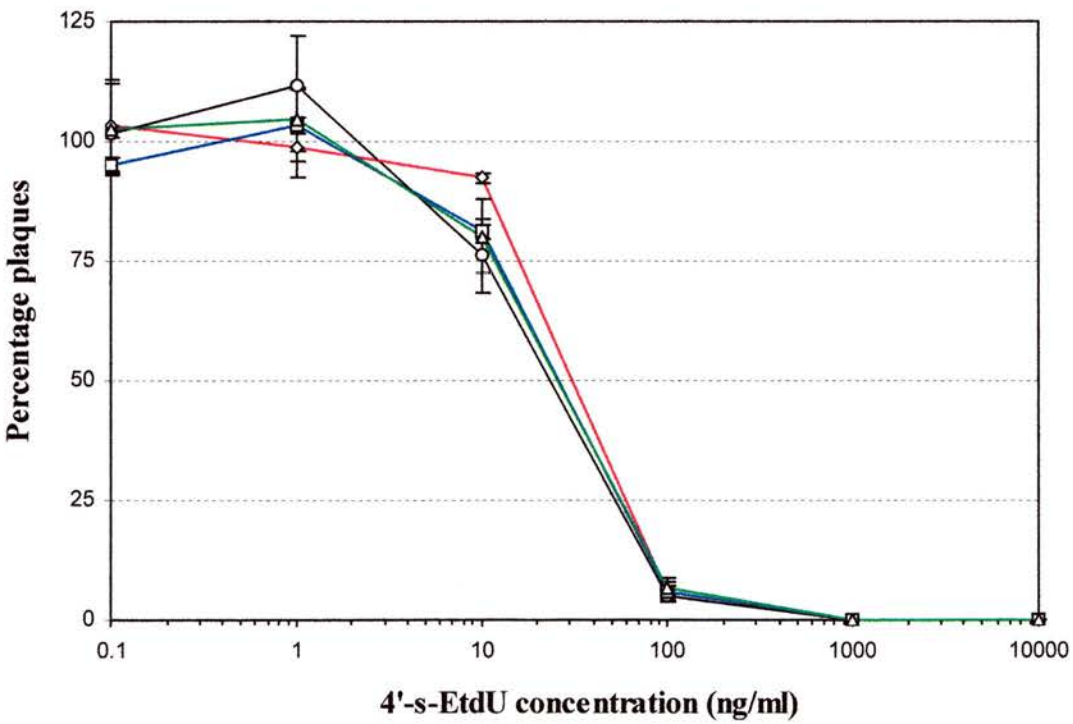

125
100
75
50
25
0
Percentage plaques
0.1
1
10
100
1000
10000
4'-s-EtdU concentration (ng/ml)

D


125
100
75
50
25
0
Percentage plaques
0.1
1
10
100
1000
10000
4'-s-EtdU concentration (ng/ml)

E

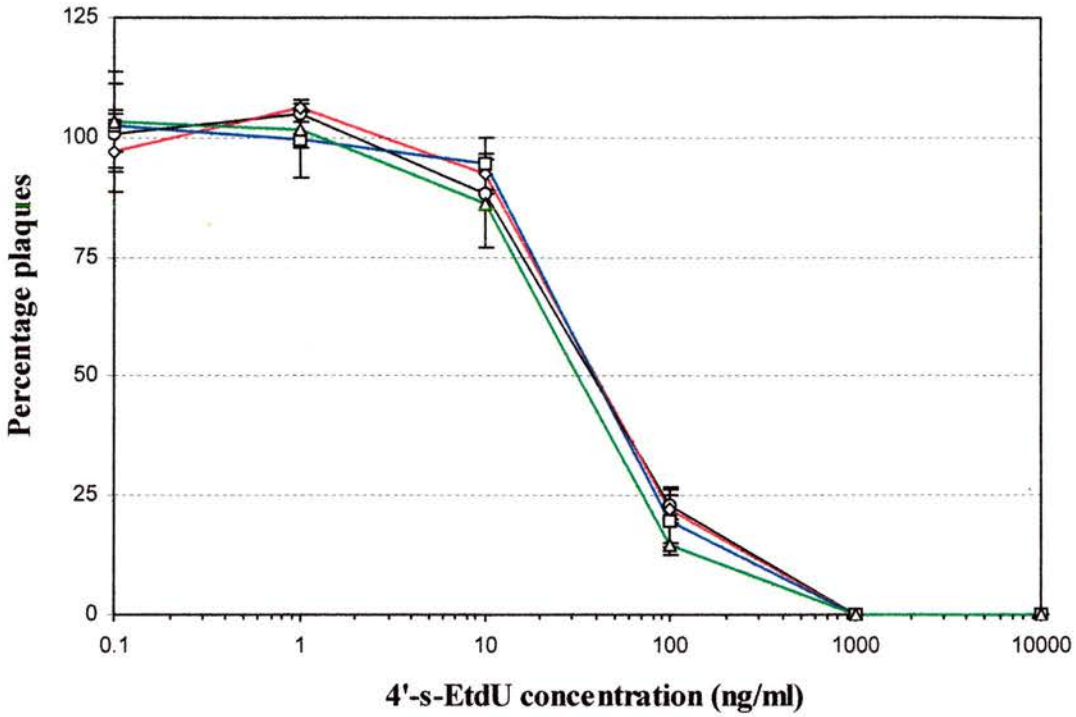


Figure 4.1.6 The sensitivity of different virus isolate to 4'-s-EtdU, reactivated from of the lungs (A and B) and splenocytes (C, D and E) removed from mice sampled on days 38 and 61 post infection, as determined by EC_{50} assay. The virus isolates were generated by lung and splenocyte co-cultivation assay, from mice that were either left untreated (black and brown), 4'-s-EtdU treated and not withdrawn (blue) or 4'-s-EtdU treated and withdrawn on day 12 post infection (red, yellow and purple). The virus isolates were compared to the original MHV-68 working stock (green). The data is represented by the mean percentage plaquing efficiency, of the virus isolates at different concentrations of 4'-s-EtdU. The error bars represent the standard deviation and the dotted lines represent 25%, 50%, and 75% plaquing efficiency levels (see Table 4.1 for further details).

Table 4.1 **The summarised results from *figure 4.1.6* (the EC_{50} assays carried out on MHV-68, isolated from both 4'-s-EtdU treated and untreated, long-term infected mice).**

Graph	Isolate Source	Graph Line Colour	Mouse number	[1] Day Sampled	[2] Crude EC_{50} (ng/ml)
A	MHV-68 working stock	Green	-	-	61.4
A	Group 1 Lung	Black	1	38	64.7
A	Group 1 Lung	Brown	2	38	56.2
A	Group 2B Lung	Red	1	38	65.5
A	Group 2B Lung	Yellow	2	38	61.5
B	MHV-68 working stock	Green	-	-	41.1
B	Group 1 Lung	Black	1	61	35.6
B	Group 1 Lung	Brown	2	61	35.5
B	Group 2B Lung	Red	1	61	41.6
B	Group 2B Lung	Yellow	2	61	40.6
B	Group 2B Lung	Pink	3	61	38.5
C	MHV-68 working stock	Green	-	-	39.8
C	Group 1 Spleen	Black	1	61	39.6
C	Group 2A Spleen	Blue	2	61	49.4
C	Group 2B Spleen	Red	1	61	36.6
D	MHV-68 working stock	Green	-	-	25.8
D	Group 1 Spleen	Black	2	61	23.2
D	Group 2A Spleen	Blue	3	61	25.9
D	Group 2B Spleen	Red	2	61	30.8
E	MHV-68 working stock	Green	-	-	32.0
E	Group 1 Spleen	Black	3	61	38.7
E	Group 2A Spleen	Blue	4	61	39.3
E	Group 2B Spleen	Red	3	61	40.1

Key: - ~ Not applicable, [1] ~ Time when mice were killed (days post infection), [2] ~ Value calculated from the graph, assuming at straight line between points either side of 50% inhibition. The **Group 1** mice were left untreated, the **Group 2** mice were treated from day 3 post infection with 4'-s-EtdU and either left treated (**A**) or withdrawn from treatment on day 12 post infection (**B**).

replication, at the site of acute infection, is necessary for the establishment of viral latency in B-lymphocytes and/or to induce disease pathology, two experiments were performed on mice treated with 4'-s-EtdU from 2 days prior to infection with MHV-68.

Twenty eight BALB/c female mice, aged 3 to 4 weeks were randomly selected from a batch of 48 age and sex matched animals and were put on drinking water containing 0.3mg/ml 4'-s-EtdU. The remaining 20 animals remained on normal drinking water. After 2 days of treatment, the mice on 4'-s-EtdU drinking water the normal drinking water, were infected intra-nasally with $4x10^5$ pfu MHV-68. At day 12 post infection, 7 of the mice put onto 4'-s-EtdU drinking water were withdrawn from treatment and put back onto normal drinking water. The remaining prophylactically treated mice remained on the 4'-s-EtdU drinking water for the duration of the experiment. The mice were sampled at days 3, 7, 12, 21 and 31 days post infection.

4.2.4 **The effect of prophylactic 4'-s-EtdU treatment on MHV-68 infection of the lungs of mice.**

Lungs were removed from all animals at all time points and infectious virus assays carried out on the homogenates. Lung co-cultivation assays were also performed on all lung tissues except for those from days 3 and 7 post infection. An acute viral infection of the lungs occurred in the untreated mice following the intra-nasal inoculation of MHV-68. Relatively high titres of infectious virus could be detected in the lungs of the untreated mice at both days 3 and 7 post infection. However, no infectious virus was detectable by direct plaque assay, in the lungs of the prophylactically 4'-s-EtdU treated mice at any time point tested, including those normally associated with the acute infection (*see figure 4.2.1*).

Virus was reactivated from the lungs of all the untreated mice sampled at days 12, 21 and 31 post infection, by co-cultivation assay. Virus was also reactivated from the

Figure 4.2.1

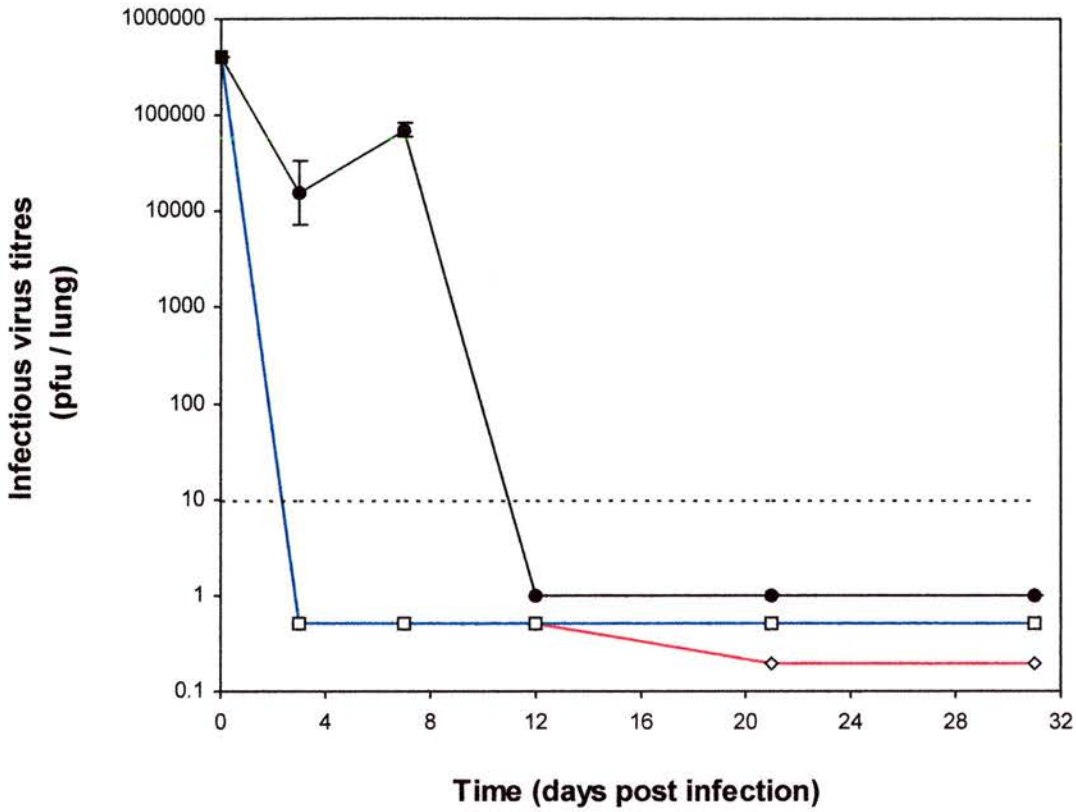


Figure 4.2.1 The infectious virus titres (as measured by direct plaque assay) detected in the lungs of mice infected intra-nasally with $4x10^5$ pfu MHV-68. The mice were either left untreated (black), prophylactically 4'-s-EtdU treated and not withdrawn (blue) or withdrawn from treatment day 12 post infection (red). Four mice were sampled from each group at every time point (days 3, 7, 12 and 21 post infection) except day 31 post infection. Four mice were sampled from the untreated group at day 31 post infection but only 3 mice from the prophylactically treated (and not withdrawn) group and the treatment withdrawn (at day 12 post infection) group. The values are represented by geometric means and the error bars represent the standard deviation. The dotted line represents the assay limit of detection.

lungs of the mice withdrawn from the 4'-s-EtdU treatment, at both days 21 and 31 post infection. However, virus could only be reactivated from 2/11 mice not withdrawn from treatment, 2/4 at the earliest time point, day 12 post infection, and 0/7 at the later 2 time points, days 21 and 31 post infection (*see figure 4.2.2*).

4.2.5 **The effect of prophylactic 4'-s-EtdU treatment on the MHV-68 induced splenomegaly in mice.**

Spleens were taken from all mice, except those sampled at day 3 post infection. The untreated mice experienced a splenomegaly (*see figure 4.2.3*). At day 7 post infection, there was no significant difference between the mean number of splenocytes observed in treated and untreated mice (P= 0.7 by student T-test). However, the mean number of splenocytes observed in the untreated mice had significantly increased by day 12 post infection. The splenocyte counts were still significantly elevated at day 21 post infection but had decreased to a non-significantly elevated level by day 31 post infection (P= 0.002, 0.004 and 0.15 respectively, by student T-test).

The prophylactically 4'-s-EtdU treated mice (both withdrawn and non-withdrawn from treatment) did not experience splenomegaly, with the mean number of splenocytes showing no significant change across the time course. There was no significant differences between the mean number of splenocytes observed in the mice withdrawn and not withdrawn from treatment. The 4'-s-EtdU treatment did not appear to directly affect the number of splenic leukocytes observed in the mice, since at day 7 post infection there was no significant difference between the treated and untreated mouse groups. However, due to the splenomegaly experienced by the untreated mice, the mean number of splenocytes was significantly elevated as compared to the treated and not withdrawn mice, at days 12 and 21, and the mice withdrawn from treatment, at day 21 post infection (P= 0.0024, 0.015 and 0.022 respectively, by student T-test). By day 31 post infection, the splenomegaly in the untreated mice had receded and there was no longer a significant difference in the

Figure 4.2.2

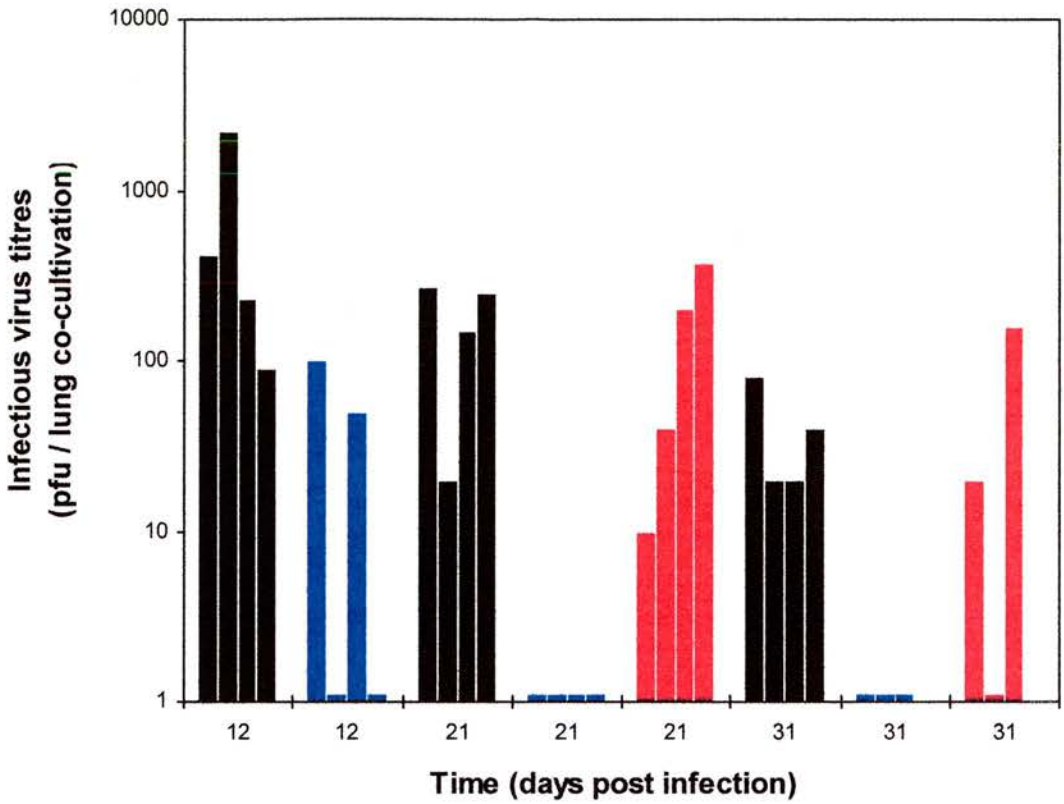


Figure 4.2.2 The levels of infectious virus reactivated (by co-cultivation assay) from the lung tissue of mice infected intra-nasally with $4x10^5$ pfu MHV-68. The mice were either left untreated (black), prophylactically 4'-s-EtdU treated and not withdrawn (blue) or withdrawn from treatment day 12 post infection (red). Four mice were sampled from each group at every time point (days 3, 7, 12 and 21 post infection) except day 31 post infection. Four mice were sampled from the untreated group at day 31 post infection but only 3 mice from the prophylactically treated (and not withdrawn) group and the treatment withdrawn (at day 12 post infection) group. The assay limit of detection was 5 pfu per co-cultivation.

Figure 4.2.3

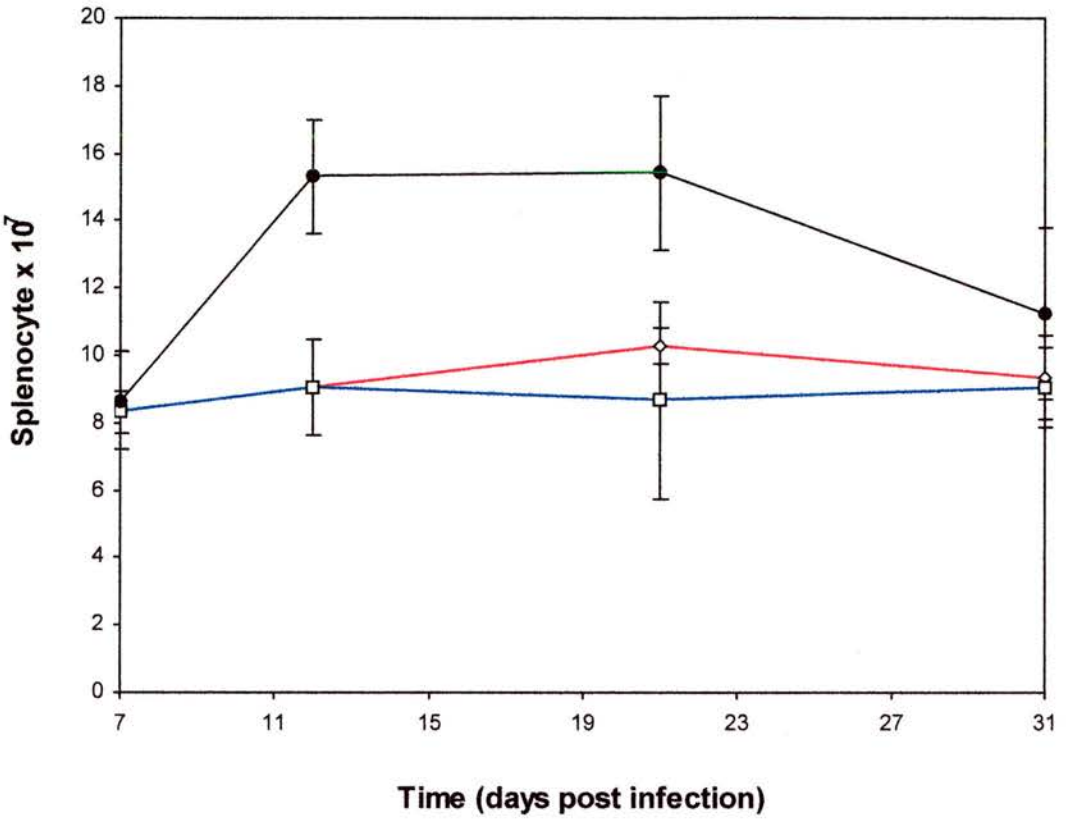


Figure 4.2.3 The number of splenocytes observed in mice infected intra-nasally with $4x10^5$ pfu MHV-68. The mice were either left untreated (black), prophylactically 4'-s-EtdU treated and not withdrawn (blue) or withdrawn from treatment day 12 post infection (red). Four mice were sampled from each group at every time point (days 3, 7, 12 and 21 post infection) except day 31 post infection. Four mice were sampled from the untreated group at day 31 post infection but only 3 mice from the prophylactically treated (and not withdrawn) group and the treatment withdrawn (at day 12 post infection) group. The values are represented by arithmetic means and the error bars represent the standard deviation.

mean number of splenocytes observed in any of the three groups of mice.

4.2.6 **The effect of prophylactic 4'-s-EtdU treatment on the MHV-68 infection of the splenocytes of mice.**

The splenocyte infectious centre titre in the untreated mice followed the pattern, commonly observed in BALB/c mice, following intra-nasal infection with MHV-68. The splenic infectious centre titre was at a relatively low level at day 7 post infection. By day 12 post infection, the infectious centre had significantly increased (P=0.0003 by student T-test) to peaked levels. By day 21, the titre had significantly decreased (P=0.0002 by student T-test) back to the base level, remaining unaltered at day 31 post infection (P=0.59 by student T-test). No infectious centres were observed in any of the mice not withdrawn from the prophylactic 4'-s-EtdU treatment, at any time point. An infectious centre titre was not detected in the mice withdrawn from the 4'-s-EtdU treatment, at day 21 post infection. However, 2 /3 mice assayed at day 31 post infection, gave rise to infectious centres, albeit on the limits of the assay sensitivity (*see figure 4.2.4*).

Because the levels of splenic reactivation were potentially very low, for the mice withdrawn and not withdrawn from the prophylactic 4'-s-EtdU treatment withdrawn, splenocyte co-cultivation assays were performed, as well as by infectious centre assays. The splenocyte co-cultivation assays confirmed that virus could be reactivated from 2/3 mice withdrawn from treatment, sampled at day 31 post infection. However, no virus could be reactivated from any of the mice not withdrawn from treatment, sampled at days 12, 21 and 31 or the treatment withdrawn mice, sampled at day 21 post infection (*see Table 4.2*).

4.2.7 **The detection of MHV-68 genomic DNA in the splenocytes and lung tissue of mice treated with 4'-s-EtdU.**

Since virus could not be detected by standard reactivation assays in the

Figure 4.2.4

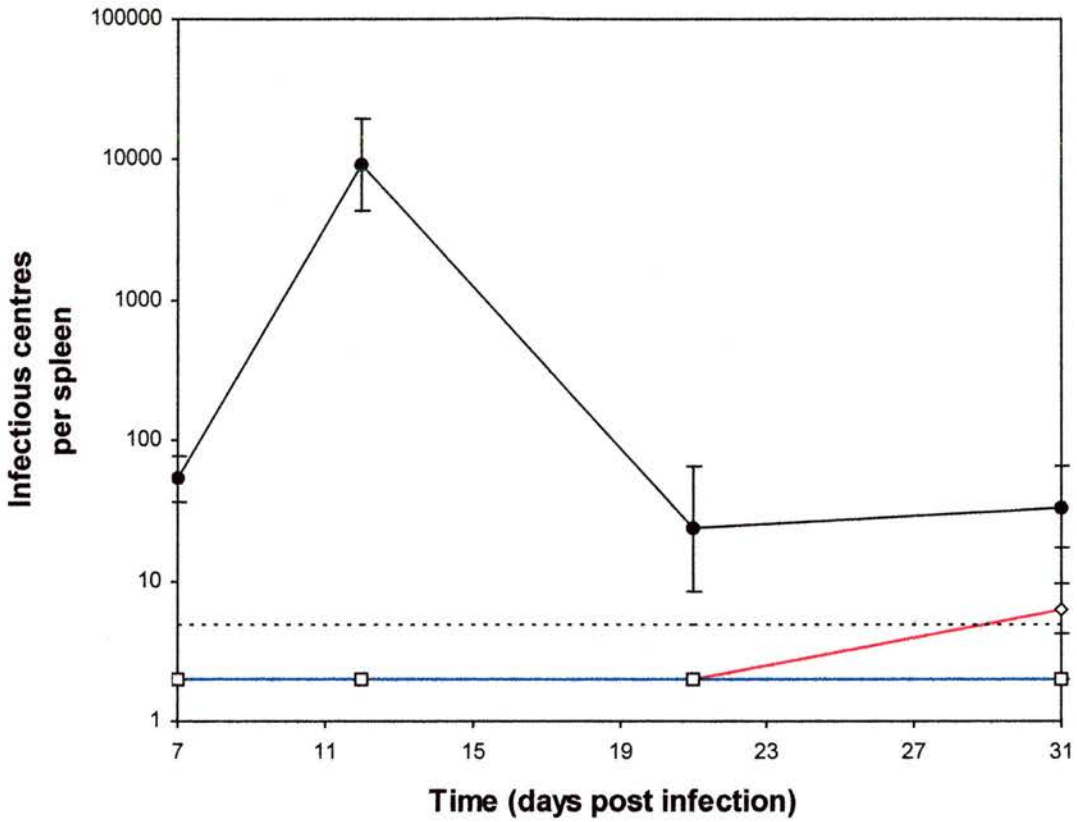


Figure 4.2.4 The latent virus titres (as measured by infectious centre assay) detected in the spleens of mice infected intra-nasally with $4x10^5$ pfu MHV-68. The mice were either left untreated (black), prophylactically 4'-s-EtdU treated and not withdrawn (blue) or withdrawn from treatment day 12 post infection (red). Four mice were sampled from each group at every time point (days 3, 7, 12 and 21 post infection) except day 31 post infection. Four mice were sampled from the untreated group at day 31 post infection but only 3 mice from the prophylactically treated (and not withdrawn) group and the treatment withdrawn (at day 12 post infection) group. The values are represented by geometric means and the error bars represent the standard deviation. The dotted line represents the assay limit of detection.

Table 4.2 **The effect of prophylactic 4'-s-EtdU treatment on the MHV-68 infectious virus titres reactivated from splenocytes (by co-cultivation assay) of intra-nasally infected mice.**

Mouse Number	Infectious virus titre (pfu) per splenocyte co-cultivation					
	Day 21 post infection			Day 31 post infection		
	Untreated	Treated	Withdrawn	Untreated	Treated	Withdrawn
1	1600	< 5	< 5	1800	< 5	< 5
2	ND	< 5	< 5	3000	< 5	950
3	ND	< 5	< 5	2000	< 5	5900
4	ND	<5	<5	8800	-	-

Key: ND - Not done.

prophylactically 4'-s-EtdU treated mice, MHV-68 specific PCR was carried out on DNA extracted from both splenocytes and lung tissue from the mice sampled at the later experimental time points. The virus genomic DNA could not be detected in DNA extracted from the splenocytes of the continually 4'-s-EtdU treated mice. First round (40 cycle) PCR carried out on DNA extracted from the splenocytes of the untreated mice, produced a MHV-68 specific band of 500bp with 3/4 mice sampled at day 21 post infection and 2/3 mice sampled at day 31 post infection. However, none of the treated mice either those withdrawn (0/7) or not withdrawn from treatment (0/7), sampled at both days 21 and 31 post infection, gave rise to a MHV-68 specific band of 500bp, after 40 cycles of PCR (*see figure 4.2.5*). After a further 25 cycles of nested PCR, the DNA extracted from the splenocyte samples, from all the untreated mice, sampled at both days 21 (4/4) and 31 (3/3) post infection, gave rise to a MHV-68 specific nested PCR band of 400bp. Of the mice withdrawn from treatment, only 1/4 splenocyte DNA samples, from the mice sampled at day 21 post infection, gave rise to a MHV-68 specific nested PCR band of 400bp. However, by day 31 post infection, all splenocyte DNA samples, derived from the mice withdrawn from treatment, gave rise to MHV-68 specific nested PCR bands at 400bp. Nested PCR carried out on splenocyte DNA, derived from the treated mice that were not withdrawn from treatment, sampled on both days 21 and 31 post infection, failed to produced MHV-68 specific bands (0/4 and 0/3 respectively) (*see figure 4.2.6*).

Contrary to the results from the PCR analysis carried out on the splenocyte DNA, the lung tissue of all the mice, from all 3 groups, sampled at day 31 post infection, were shown to harbour MHV-68 genomic DNA. DNA extracted from the lung tissue of the untreated mice (4/4) and mice withdrawn from treatment (3/3), sampled at day 31 post infection, gave a MHV-68 specific PCR band of 500bp, after 40 cycles of PCR (*see figure 4.2.7*). The DNA extracted from the lung tissue of all the treated mice that were not withdrawn from treatment, failed to produce a MHV-68 specific band after 40 cycles of PCR (0/3). However all the mice not withdrawn from treatment (3/3) went on to produce a virus specific PCR band at 400bp, after a

Figure 4.2.5

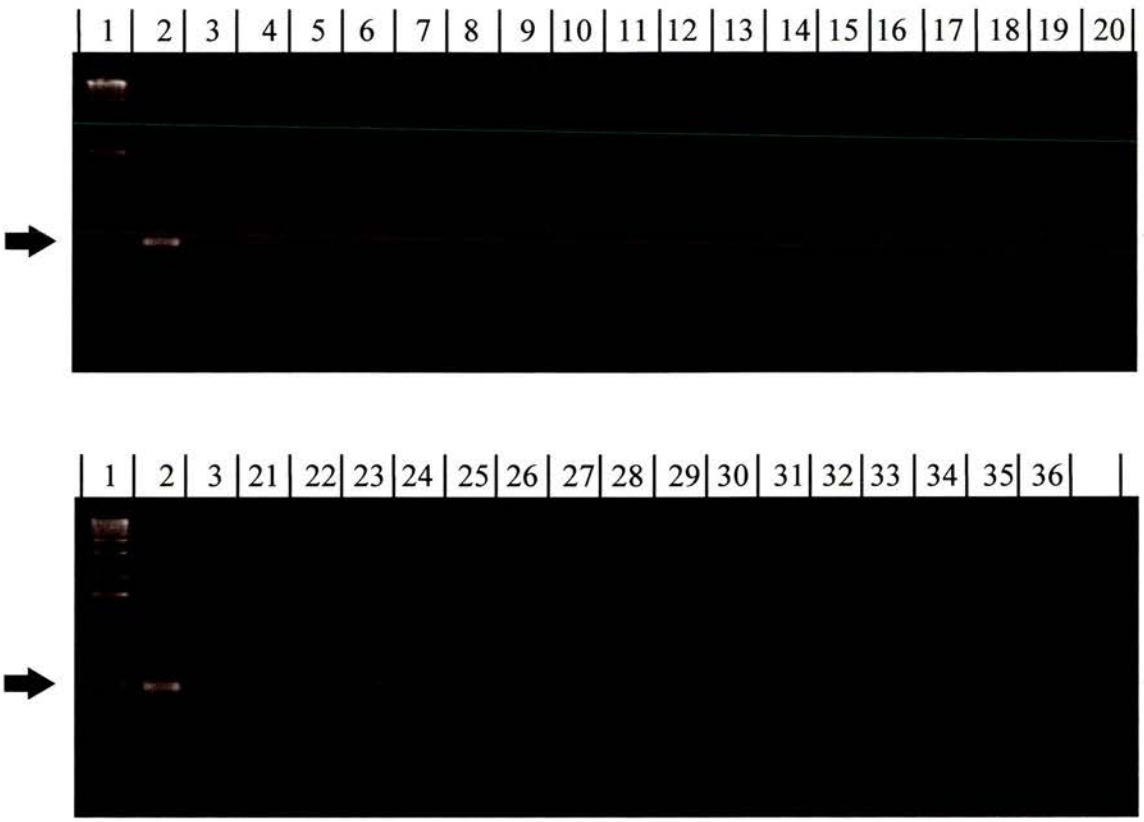


Figure 4.2.5 The effects of prophylactic 4'-s-EtdU treatment on the establishment of viral persistance in the splenocytes of mice infected infected by the intra-nasal and intra-peritoneal routes of infection, as determined by first round PCR. Four cycles of MHV-68 specific PCR was carried out on 500ng of DNA extracted from the lung tissue of mice which were either either infected intra-nasally MHV-68 and sampled at day 21 (lanes 6 to 15) and day 31 post infection (lanes 29 to 37) or infected intra-peritoneally and sampled at day 24 post infection (lanes 16 to 28). The infected mice were either left untreated (lanes 4 to 7, 16 to 20 and 29 to 31), prophylactically treated with 4'-s-EtdU and not withdrawn (lanes 12 to 15, 25 to 28 and 35 to 37) or withdrawn from treatment at day 12 post infection (lanes 8 to 11, 21 to 24 and 32 to 34). Lane 2 represents the PCR positive control and was carried out on 200ng of DNA extracted from MHV-68 persistently infected NS0 cells. Lane 3 represent the PCR negative control and was carried out on 500ng of DNA extracted from splenocytes of an uninfected BALB/c mouse. The PCR products were visualised by 2% agarose gel electrophoresis and ethidium bromide staining, viewed under UV. Product size was determined by comparison to 1kbp Ladder (lane 1). For further details see table4.3.

Table 4.3 **The summarised results in *figure 4.2.5* (the first round MHV-68 specific PCR, carried out on splenic DNA extracted from both 4'-s-EtdU treated and untreated, MHV-68 infected mice).**

Lane Number	Sample Description	Mouse Number	Route of MHV-68 Inoculation	[1] Day Sampled	PCR Result
1	1KB Ladder	-	-	-	-
2	[2] Positive Control	-	-	-	+ve
3	[3] Negative Control	-	-	-	-ve
4	Group 1 spleen	1	Intra-nasal	21	+ve
5	Group 1 spleen	2	Intra-nasal	21	-ve
6	Group 1 spleen	3	Intra-nasal	21	+ve
7	Group 1 spleen	4	Intra-nasal	21	+ve
8	Group 2B spleen	1	Intra-nasal	21	-ve
9	Group 2B spleen	2	Intra-nasal	21	-ve
10	Group 2B spleen	3	Intra-nasal	21	-ve
11	Group 2B spleen	4	Intra-nasal	21	-ve
12	Group 2A spleen	1	Intra-nasal	21	-ve
13	Group 2A spleen	2	Intra-nasal	21	-ve
14	Group 2A spleen	3	Intra-nasal	21	-ve
15	Group 2A spleen	4	Intra-nasal	21	-ve
16	Group 1 spleen	1	Intra-peritoneal	24	+ve
17	Group 1 spleen	2	Intra-peritoneal	24	+ve
18	Group 1 spleen	3	Intra-peritoneal	24	+ve
19	Group 1 spleen	4	Intra-peritoneal	24	+ve
20	Group 1 spleen	5	Intra-peritoneal	24	+ve
21	Group 2B spleen	1	Intra-peritoneal	24	-ve
22	Group 2B spleen	2	Intra-peritoneal	24	+ve
23	Group 2B spleen	3	Intra-peritoneal	24	-ve
24	Group 2B spleen	4	Intra-peritoneal	24	-ve
25	Group 2A spleen	1	Intra-peritoneal	24	-ve
26	Group 2A spleen	2	Intra-peritoneal	24	-ve
27	Group 2A spleen	3	Intra-peritoneal	24	-ve
28	Group 2A spleen	4	Intra-peritoneal	24	-ve
29	Group 1 spleen	1	Intra-nasal	31	+ve
30	Group 1 spleen	2	Intra-nasal	31	-ve
31	Group 1 spleen	3	Intra-nasal	31	+ve
32	Group 2B spleen	1	Intra-nasal	31	-ve
33	Group 2B spleen	2	Intra-nasal	31	-ve
34	Group 2B spleen	3	Intra-nasal	31	-ve
35	Group 2A spleen	1	Intra-nasal	31	-ve
36	Group 2A spleen	2	Intra-nasal	31	-ve
37	Group 2A spleen	3	Intra-nasal	31	-ve

Key: **-** ~ Not applicable, **-ve** ~ PCR negative, **+ve** ~ PCR positive, [1] ~ Time when mice were killed (days post infection), [2] ~ DNA extracted from MHV-68 infected NS0 cells, [3] ~ DNA extracted from uninfected NS0 cells. The **Group 1** mice were left untreated, the **Group 2** mice were treated prophylactically with 4'-s-EtdU and either not withdrawn (**A**) or withdrawn from treatment on day 12 post infection (**B**).

Figure 4.2.6

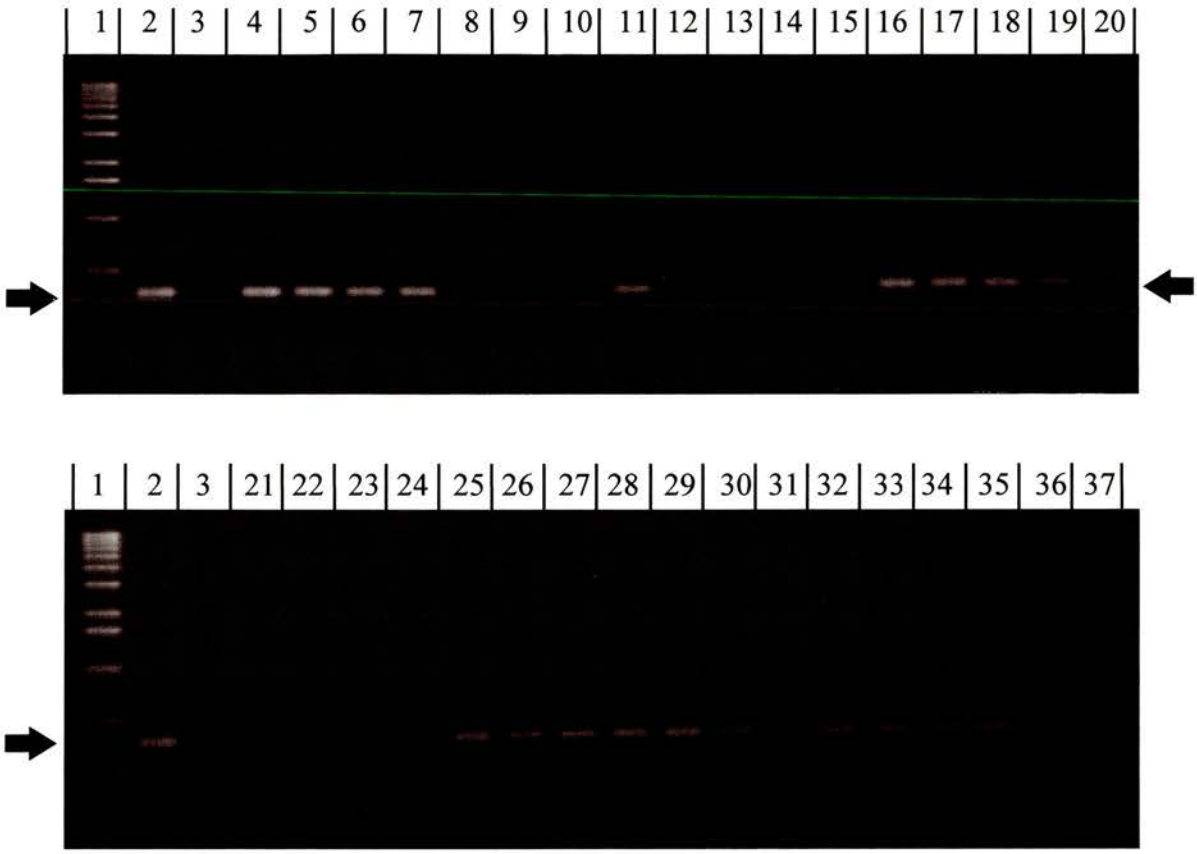


Figure 4.2.6 The effects of prophylactic 4'-s-EtdU treatment on the establishment of viral persistance in the splenocytes of mice infected infected by the intra-nasal and intra-peritoneal routes of infection, as determined by nested PCR. A firther 25 cycle MHV-68 specific nested PCR carried out on 2μl of the products from the first round PCR, originally carried out on 500ng of DNA extracted from splenocytes of MHV-68 infected mice. The mice were either infected intra-nasally MHV-68 and sampled at day 21 (lanes 4 to 15) and day 31 post infection (lanes 16 to 24) or infected intra-peritoneally and sampled at day 24 post infection (lanes 25 to 37). The infected mice were either left untreated (lanes 4 to 7, 16 to 18 and 25 to 29), prophylactically treated with 4'-s-EtdU and not withdrawn (lanes 12 to 15, 22 to 24 and 34 to 37) or withdrawn from treatment at day 12 post infection (lanes 8 to 11, 19 to 21 and 32 to 34). Lane 2 represents the PCR positive control and was carried out on 200ng of DNA extracted from MHV-68 persistently infected NS0 cells. Lane 3 represent the PCR negative control and was carried out on 500ng of DNA extracted from splenocytes of an uninfected BALB/c mouse. The nest PCR products were visualised by 2% agarose gel electrophoresis and ethidium bromide staining, viewed under UV. Product size was determined by comparison to 1kbp Ladder (lane 1). For further information see Table 4.4.

Table 4.4 **The summarised results in *figure 4.2.6* (the MHV-68 specific nested PCR, carried out on splenic DNA extracted from both 4'-s-EtdU treated and untreated MHV-68 infected mice).**

Lane Number	Sample Description	Mouse Number	Route of MHV-68 Inoculation	[1] Day Sampled	Nested PCR Result
1	1KB Ladder	-	-	-	-
2	[2] Positive Control	-	-	-	+ve
3	[3] Negative Control	-	-	-	-ve
4	Group 1 spleen	1	Intra-nasal	21	+ve
5	Group 1 spleen	2	Intra-nasal	21	+ve
6	Group 1 spleen	3	Intra-nasal	21	+ve
7	Group 1 spleen	4	Intra-nasal	21	+ve
8	Group 2B spleen	1	Intra-nasal	21	-ve
9	Group 2B spleen	2	Intra-nasal	21	-ve
10	Group 2B spleen	3	Intra-nasal	21	-ve
11	Group 2B spleen	4	Intra-nasal	21	+ve
12	Group 2A spleen	1	Intra-nasal	21	-ve
13	Group 2A spleen	2	Intra-nasal	21	-ve
14	Group 2A spleen	3	Intra-nasal	21	-ve
15	Group 2A spleen	4	Intra-nasal	21	-ve
16	Group 1 spleen	1	Intra-nasal	31	+ve
17	Group 1 spleen	2	Intra-nasal	31	+ve
18	Group 1 spleen	3	Intra-nasal	31	+ve
19	Group 2B spleen	1	Intra-nasal	31	+ve
20	Group 2B spleen	2	Intra-nasal	31	+ve
21	Group 2B spleen	3	Intra-nasal	31	+ve
22	Group 2A spleen	1	Intra-nasal	31	-ve
23	Group 2A spleen	3	Intra-nasal	31	-ve
24	Group 2A spleen	2	Intra-nasal	31	-ve
25	Group 1 spleen	1	Intra-peritoneal	24	+ve
26	Group 1 spleen	2	Intra-peritoneal	24	+ve
27	Group 1 spleen	3	Intra-peritoneal	24	+ve
28	Group 1 spleen	4	Intra-peritoneal	24	+ve
29	Group 1 spleen	5	Intra-peritoneal	24	+ve
30	Group 2B spleen	1	Intra-peritoneal	24	+ve
31	Group 2B spleen	2	Intra-peritoneal	24	+ve
32	Group 2B spleen	3	Intra-peritoneal	24	+ve
33	Group 2B spleen	4	Intra-peritoneal	24	+ve
34	Group 2A spleen	1	Intra-peritoneal	24	+ve
35	Group 2A spleen	2	Intra-peritoneal	24	+ve
36	Group 2A spleen	3	Intra-peritoneal	24	-ve
37	Group 2A spleen	4	Intra-peritoneal	24	-ve

Key: - ~ Not applicable, **-ve** ~ nested PCR negative, **+ve** ~ nested PCR positive, [1] ~ Time when mice were killed (days post infection), [2] ~ DNA extracted from MHV-68 infected NS0 cells, [3] ~ DNA extracted from uninfected NS0 cells. The **Group 1** mice were left untreated, the **Group 2** mice were treated prophylactically with 4'-s-EtdU and either not withdrawn (**A**) or withdrawn from treatment on day 12 post infection (**B**).

Figure 4.2.7

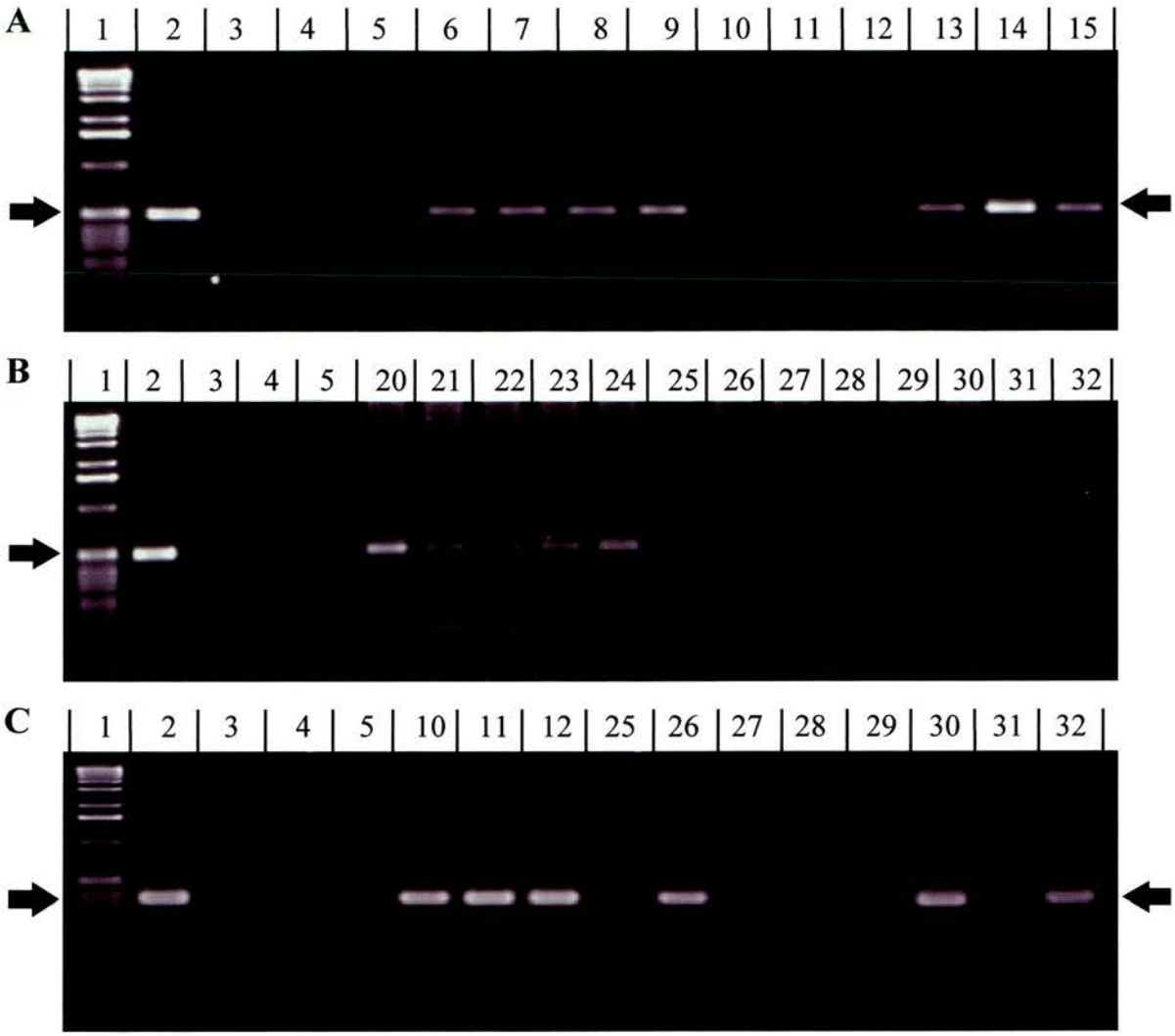


Figure 4.2.7 The effects of prophylactic 4'-s-EtdU treatment on the establishment of viral persistence in the lungs of mice infected infected by the intra-nasal and intra-peritoneal routes of infection. First round MHV-68 specific PCR was carried out on 500ng of DNA extracted from the lung tissue of mice which were either infected intra-nasally and sampled at day 31 post infection (A) or infected intra-peritoneally and sampled at day 24 post infection (B). A further 25 cycles of MHV-68 specific nested PCR was also carried out on 10 μl of the first round PCR products that failed to produce a virus spesific band at 500bp (C). The infected mice were either left untreated (lanes 6 to 9 and 20 to 24), prophylactically treated with 4'-s-EtdU and not withdrawn (lanes 10 to 12 and 25 to 28) or withdrawn from treatment at day 12 post infection (lanes 13 to 15 and 29 to 32). Lane 2 represents the PCR positive control and was carried out on 200ng of DNA extracted from MHV-68 persistently infected NS0 cells. Lane 3 represents a PCR blank control and was carried out on 10μl of distilled water. Lanes 4 and 5 represent PCR negative controls and were carried out on 500ng of DNA extracted from lung tissue of uninfected BALB/c mice. The products were visualised by 2% agarose gel electrophoresis and ethidium bromide staining, viewed under UV. Product size was determined by comparison to 1kbp Ladder (lane 1). For further information see Table 4.5.

Table 4.5 **The summarised results in *figure 4.2.7* (the first round and nested MHV-68 specific PCR, carried out on lung DNA extracted from both 4'-s-EtdU treated and untreated MHV-68 infected mice).**

Lane Number	Sample Description	Mouse Number	Route of MHV-68 Inoculation	[1] Day Sampled	1st Round PCR Results	Nested PCR Result
1	1KB Ladder	-	-	-	-	-
2	[2] Positive Control	-	-	-	+ve	+ve
3	[3] Blank	-	-	-	-ve	-ve
4	Uninfected Lung	1	-	-	-ve	-ve
5	Uninfected Lung	2	-	-	-ve	-ve
6	Group 1 lung	1	Intra-nasal	31	+ve	ND
7	Group 1 lung	2	Intra-nasal	31	+ve	ND
8	Group 1 lung	3	Intra-nasal	31	+ve	ND
9	Group 1 lung	4	Intra-nasal	31	+ve	ND
10	Group 2A lung	1	Intra-nasal	31	-ve	+ve
11	Group 2A lung	2	Intra-nasal	31	-ve	+ve
12	Group 2A lung	3	Intra-nasal	31	-ve	+ve
13	Group 2B lung	1	Intra-nasal	31	+ve	ND
14	Group 2B lung	2	Intra-nasal	31	+ve	ND
15	Group 2B lung	3	Intra-nasal	31	+ve	ND
20	Group 1 lung	1	Intra-peritoneal	24	+ve	ND
21	Group 1 lung	2	Intra-peritoneal	24	+ve	ND
22	Group 1 lung	3	Intra-peritoneal	24	+ve	ND
23	Group 1 lung	4	Intra-peritoneal	24	+ve	ND
24	Group 1 lung	5	Intra-peritoneal	24	+ve	ND
25	Group 2A lung	1	Intra-peritoneal	24	-ve	-ve
26	Group 2A lung	2	Intra-peritoneal	24	-ve	+ve
27	Group 2A lung	3	Intra-peritoneal	24	-ve	-ve
28	Group 2A lung	4	Intra-peritoneal	24	-ve	-ve
29	Group 2B lung	1	Intra-peritoneal	24	-ve	-ve
30	Group 2B lung	2	Intra-peritoneal	24	+ve	+ve
31	Group 2B lung	3	Intra-peritoneal	24	-ve	-ve
32	Group 2B lung	4	Intra-peritoneal	24	-ve	+ve

Key: **-** ~ Not applicable, **ND** ~ sample not tested, **-ve** ~ negative, **+ve** ~ positive, [1] ~ Time when mice were killed (days post infection), [2] ~ DNA extracted from MHV-68 infected NS0 cells, [3] ~ sterile distilled water. The **Group 1** mice were left untreated, the **Group 2** mice were treated prophylactically with 4'-s-EtdU and either not withdrawn (**A**) or withdrawn from treatment on day 12 post infection (**B**).

further 25 cycles of nested PCR (*see figure 4.2.7c*).

4.2.8 **The long-term effects of prophylactic 4'-s-EtdU treatment on the MHV-68 infection.**

A second experiment was set up to determine whether MHV-68 could persist in the lungs long-term in the apparent absence of both productive viral replication and latently infected circulating B-lymphocytes. It was also of interest to determine whether a specific immune response was made to MHV-68 late antigens in the prophylactically 4'-s-EtdU treated mice.

Thirty female BALB/c mice, 3 to 4 weeks old, were randomly selected from 50 and put on drinking water containing 0.3mg/ml 4'-s-EtdU. After 24 hours treatment 4 mice on the 4'-s-EtdU drinking water and 4 of the 20 mice on the normal drinking water were tail bled and the sera stored at -30°C. After 48 hours, 9 of the untreated and 24 of the treated mice were infected with $4x10^5$ pfu MHV-68 via the intra-nasal route. At day 6 post infection, 3 mice from each of the infected groups of mice and 2 mice from the uninfected groups of mice were killed, heart bled and had their lungs and spleens removed. The lung tissue and sera were stored at -80°C and -30°C respectively. Infectious centre assays were carried out on the splenocytes. At day 12 post infection, 9 of the prophylactically 4'-s-EtdU treated mice were withdrawn from the treatment and restored to normal drinking water. At day 18 post infection, 4 mice from each group were killed and spleens, lungs and sera removed and assayed, as on day 6 post infection. On day 51 post infection, 4 of the 8 remaining 4'-s-EtdU treated infected mice were withdrawn from treatment and restored to normal drinking water. On day 54 post infection the experiment had to be terminated since the mice treated with 4'-s-EtdU for 51 days or more, both infected and uninfected, had developed peritoneal swelling. The swelling apon post-mortem examination, was attributed to ascites fluid build up. All the mice from all the groups were therefore killed, heart bled and spleens and lungs removed for analysis.

4.2.9 **The long-term effects of prophylactic 4'-s-EtdU treatment on the production MHV-68 specific antibodies by infected mice.**

Concurrent with the hypothesis that high dose 4'-s-EtdU treatment completely inhibits productive virus replication *in vivo*, the prophylactically treated mice failed to produce significant levels of MHV-68 late antigen specific antibodies, following intra-nasal infection. Whole virus lysate ELISA was carried out on the sera collected from the mice sampled across the time course. The background level was determined by the mean OD_{490} of the 4'-s-EtdU treated and untreated mouse bleeds taken prior to infection. Significant MHV-68 specific antibody production was determined, at the individual level, by a mouse sera giving an OD_{490} of greater than twice the background level. At the group level, significant MHV-68 specific antibody production was determined by OD_{490} comparison to the pre-infected sera, using student T-test (*see figure 4.3.1*).

At day 6 post infection none of the infected mice, treated or untreated had produced significant levels of MHV-68 specific antibodies. By day 18 post infection, all the untreated infected mice sera gave ELISA readings significantly higher than the pre-bleed values ($P=0.011$ by student T-test). However, the sera from the prophylactically treated infected mice (that were not withdrawn from treatment) remained non-significantly different from the pre-bleed sera ($P=0.3$ by student T-test). The infected mice withdrawn from the 4'-s-EtdU treatment on day 12, were border line with respect to MHV-68 specific antibody production. Of the 4 mice tested, 2 had exactly twice the background pre-bleed OD_{490} value and 2 were below. However, as a group, the OD_{490} values were significantly higher than the pre-bleed values ($P=0.002$ by student T-test) and therefore the mice were judged, as a group, to have started to produce significant levels of anti-MHV-68 antibodies. At day 54 post infection, the ELISA readings for the untreated, infected mice further increased, from the day 18 post infection values ($P=0.0002$ by student T-test). The ELISA readings for the infected mice withdrawn from treatment on day 12 post infection, also significantly increased, with all 5 animals clearly producing significant levels of

Figure 4.3.1

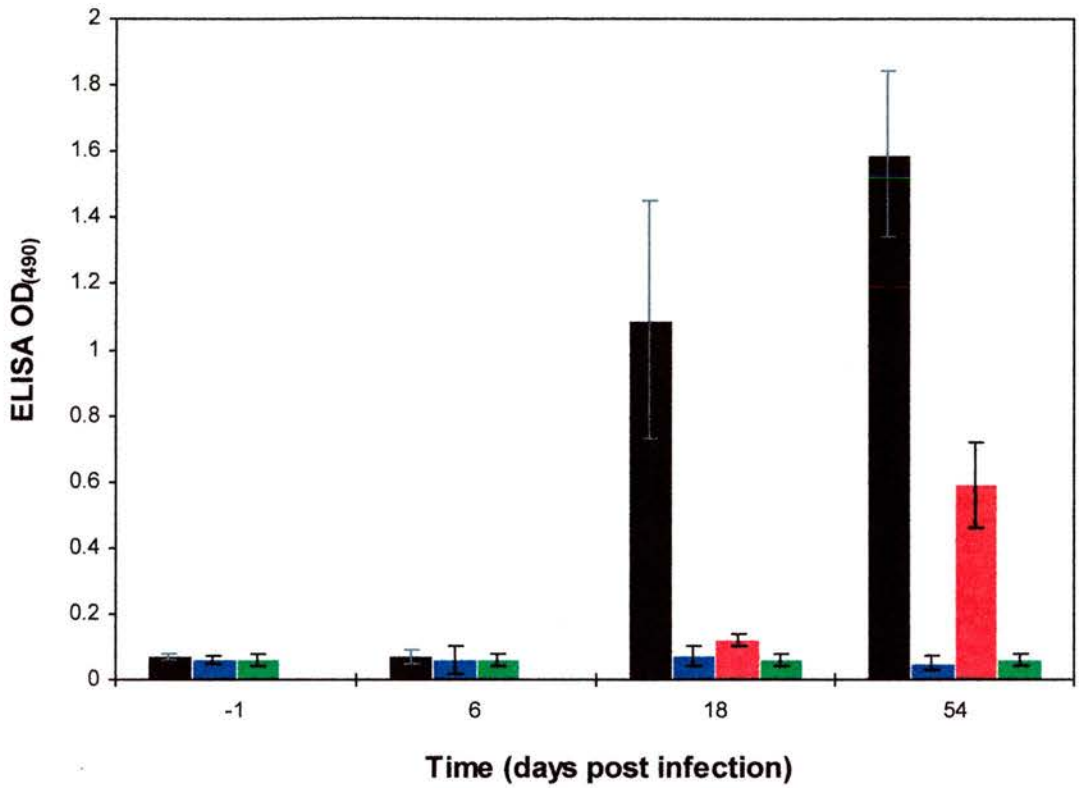


Figure 4.3.1 levels of antibody production to late MHV-68 antigens (as measured by whole virus ELISA) in mice infected intra-nasally with $4x10^5$ pfu MHV-68. The mice were either left untreated (black), prophylactically 4'-s-EtdU treated and not withdrawn (blue) or withdrawn from treatment at day 12 post infection (red). The sera from the infected mice was compared to sera from mock infected mice (green), which were bled at the same time as the infected mice. Between 3 and 4 mice from each group were bled at days –1, 6, and 18 post infection and 5 mice were bled at day 54 post infection. The values represent arithmetic means and the error bars represent the standard deviation.

anti-MHV-68 antibodies (P=0.0007 by student T-test). However, both the infected mice that were not withdrawn from treatment and the mice withdrawn from the 4'-s-EtdU treatment on day 51 post infection, remained negative with respect to MHV-68 specific antibody production (P=0.2 and 0.67 respectively by student T-test). There was no significant differences seen between the non-withdrawn 4'-s-EtdU treated mice, both infected and uninfected mice, the untreated uninfected mice, and the infected mice withdrawn from treatment on day 51 post infection.

4.3.1 **The long-term effects of prophylactic 4'-s-EtdU treatment on viral persistence in the lung**

An acute viral infection of the lung occurred in the untreated MHV-68 infected mice, indicated by the high lung infectious virus titres observed at day 6 post infection. However, no infectious virus was detected in the lungs of the prophylactically treated infected mice, at any time point, by direct plaque assay (*see figure 4.3.2*). To determine if MHV-68 genomic DNA still persisted at this site, MHV-68 specific 40 cycle PCR was carried out on DNA samples extracted from the lung tissue of the mice sampled at day 54 post infection. MHV-68 specific PCR bands at 500bp were produced by all the untreated infected mice (4/4) and 3 of the 4 treatment withdrawn (at day 12 post infection) mice. However, a MHV-68 specific PCR band was not produced by the continually treated infected mice (0/4) or treatment withdrawn (at day 51 days post infection) infected mice (0/4) (*see figure 4.3.3a*). A further 25 rounds of nested PCR was carried out on the DNA samples from the MHV-68 infected continually treated mice and the mice withdrawn from the treatment at days 12 and 51 post infection. All gave rise to MHV-68 specific nested PCR bands at 400bp (*see figure 4.3.3b*).

4.3.2 **The long-term effects of prophylactic 4'-s-EtdU treatment on splenomegaly induction and the establishment of viral latency in splenocytes.**

As with the previous experiment, the MHV-68 post acute splenomegaly occurred in

Figure 4.3.2

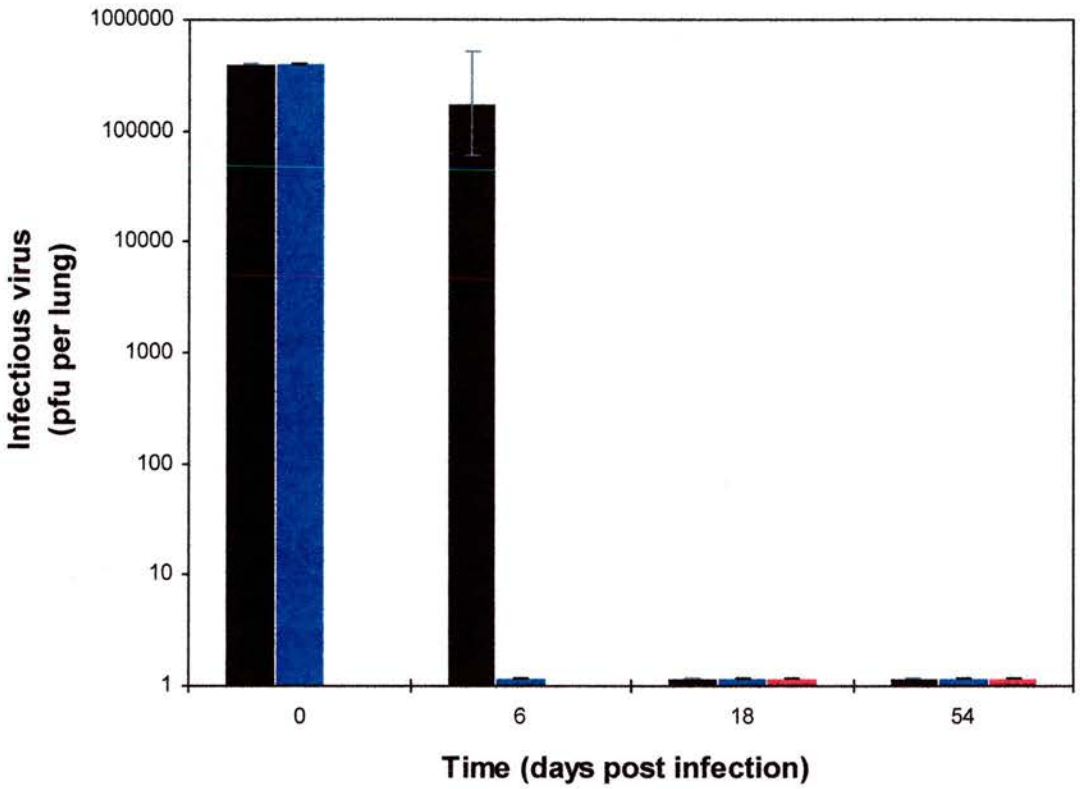


Figure 4.3.2 The infectious virus titres (as measured by direct plaque assay) detected in the lungs of mice infected intra-nasally with $4x10^5$ pfu MHV-68. The mice were either left untreated (black), prophylactically 4'-s-EtdU treated and not withdrawn (blue) or withdrawn from treatment day 12 post infection (red). Three mice were sampled from each group at day 6 and 4 mice at days 18 and 54 post infection. The values are represented by geometric means and the error bars represent the standard deviation. The assay limit of detection was 10 pfu per lung.

Figure 4.3.3

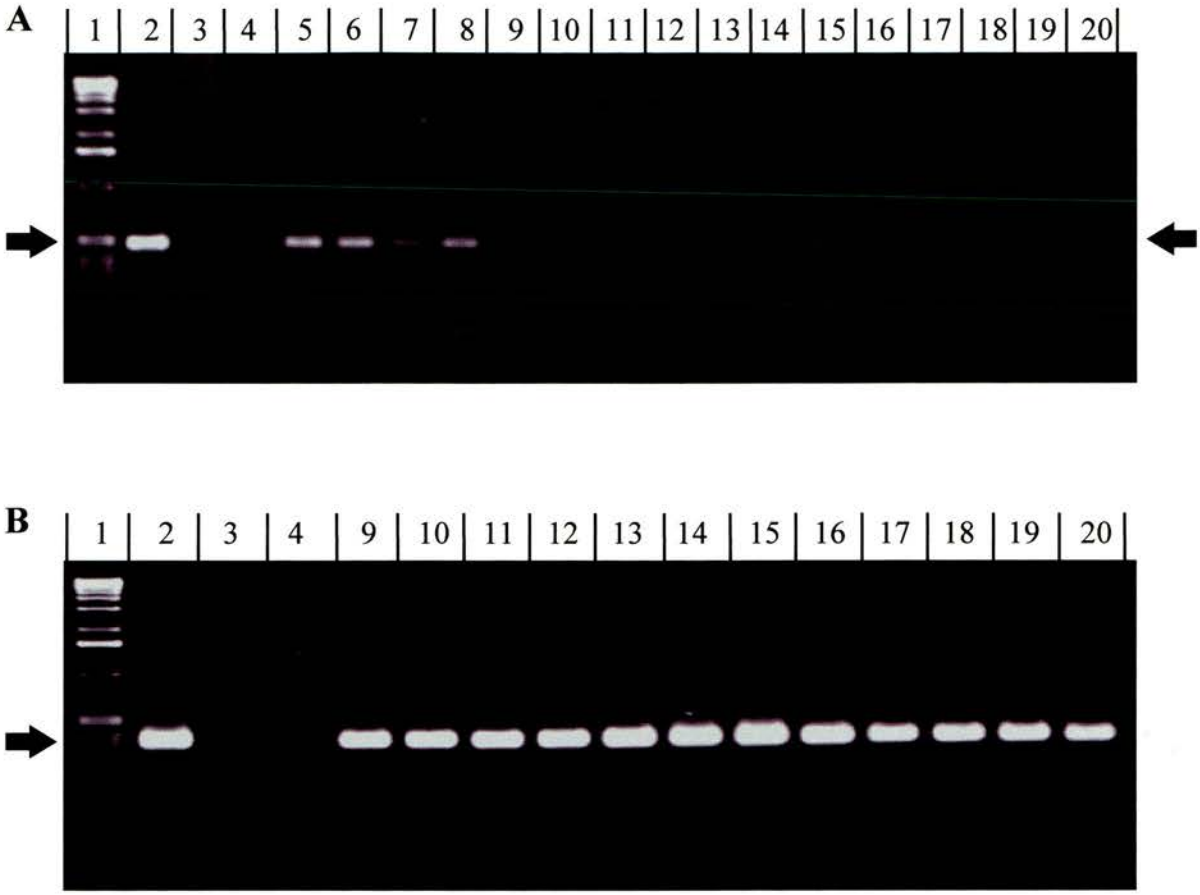


Figure 4.3.3 The long term effects of prophylactic 4'-s-EtdU treatment on the on viral persistence in the lungs of MHV-68 mice infected mice, as determined by PCR. First round MHV-68 specific PCR was carried out on 500ng of DNA extracted from the lung tissue of BALB/c mice, infected intra-nasally with $4x10^5$ pfu MHV-68 (A). A second round of 25 cycle MHV-68 spesific nested PCR was carried out on 10μl of the first round PCR products (B). The infected mice were either left untreated (lanes 5 to 8), prophylactically treated with 4'-s-EtdU and not withdrawn (lanes 9 to 12), withdrawn from treatment at 12 days post infection (lanes 13 to 16) or withdrawn from treatment at 51 days post infection (lanes 17 to 20). All mice were sampled on day 54 post infection. Lane 2 represents the PCR positive control and was carried out on 200ng of DNA extracted from MHV-68 persistently infected NS0 cells. Lanes 3 and 4 represent PCR negative controls and were carried out on 500ng of DNA extracted from lung tissue of uninfected BALB/c mice. The products were visualised by 2% agarose gel electrophoresis and ethidium bromide staining, viewed under UV. Product size was determined by comparison to 1kbp Ladder (lane 1). For further information see Table 4.6.

Table 4.6 **The summarised results in *figure 4.3.3* (the first round and nested MHV-68 specific PCR, carried out on lung DNA extracted from both 4'-s-EtdU treated and untreated, MHV-68 infected mice).**

Lane Number	Sample Description	Mouse Number	Route of MHV-68 Inoculation	[1] Day Sampled	1st Round PCR Results	Nested PCR Result
1	1KB Ladder	-	-	-	-	-
2	[2] Positive Control	-	-	-	+ve	+ve
3	Uninfected lung	1	-	-	-ve	-ve
4	Uninfected lung	2	-	-	-ve	-ve
5	Group 1 Lung	1	Intra-nasal	54	+ve	ND
6	Group 1 lung	2	Intra-nasal	54	+ve	ND
7	Group 1 lung	3	Intra-nasal	54	+ve	ND
8	Group 1 lung	4	Intra-nasal	54	+ve	ND
9	Group 2A lung	1	Intra-nasal	54	-ve	+ve
10	Group 2A lung	2	Intra-nasal	54	-ve	+ve
11	Group 2A lung	3	Intra-nasal	54	-ve	+ve
12	Group 2A lung	4	Intra-nasal	54	-ve	+ve
13	Group 2B lung	1	Intra-nasal	54	-ve	+ve
14	Group 2B lung	2	Intra-nasal	54	+ve	+ve
15	Group 2B lung	3	Intra-nasal	54	+ve	+ve
16	Group 2B lung	4	Intra-nasal	54	+ve	+ve
17	Group 2C lung	1	Intra-nasal	54	-ve	+ve
18	Group 2C lung	2	Intra-nasal	54	-ve	+ve
19	Group 2C lung	3	Intra-nasal	54	-ve	+ve
20	Group 2C lung	4	Intra-nasal	54	-ve	+ve

Key: - ~ Not applicable, **ND** ~ sample not tested, **-ve** ~ negative, **+ve** ~ positive, [1] ~ Time when mice were killed (days post infection), [2] ~ DNA extracted from MHV-68 infected NS0 cells. The **Group 1** mice were left untreated, the **Group 2** mice were treated prophylactically with 4'-s-EtdU and either not withdrawn (**A**), withdrawn from treatment on day 12 post infection (**B**) or withdrawn from treatment on day 51 post infection (**C**).

the untreated, infected mice, but failed to occur in the continuously treated and treatment withdrawn, infected mice (*see figure 4.3.4*). At day 6 post infection the numbers of splenocytes observed in the untreated infected mice was not significantly different from either the prophylactically treated infected mice or the uninfected mice (P= 0.58 and 0.48 respectively by student T test). However, there was a significant increase in the numbers of splenocytes observed in the untreated infected mice at day 18 post infection, both with respect to the numbers observed in the continually treated and treatment withdrawn infected mice and to the untreated infected mice at day 6 post infection (P= 0.0004, 0.006 and 0.0007 respectively by student T-test). By day 54 post infection, the splenomegaly in the untreated infected animals had receded. There was no significant difference between the number of splenocytes observed in the any of the different groups of mice, both infected and uninfected. No significant increase in the numbers of splenocytes was observed across the time course with the continually treated and treatment withdrawn mice.

At day 6 post infection, splenic infectious centres were detected in 2/3 untreated infected mice. By day 18 post infection, splenic infectious centres were detectable in all of the untreated infected mice, averaging 787 infectious centres per spleen. By day 54 post infection, the levels of infectious centres had decreased to an average of 9 infectious centres per spleen with infectious centres only being detectable in 2/5 mice. Infectious centres were not detectable in any of the mice not withdrawn from the prophylactic 4'-s-EtdU treatment, at any of the experimental time points. No infectious centres were detected in any of the mice withdrawn from treatment (on day 12 post infection), sampled at day 18 post infection. However, by day 54 post infectious centres were detected in 3/5 mice withdrawn from treatment, giving an average of 11 infectious centres per spleen, which was indistinguishable from the untreated mice, at the same time point (*see figure 4.3.5*). No infectious centres were detected, at day 54 post infection, in the mice withdrawn from the 4'-s-EtdU treatment on day 51 post infection.

Figure 4.3.4

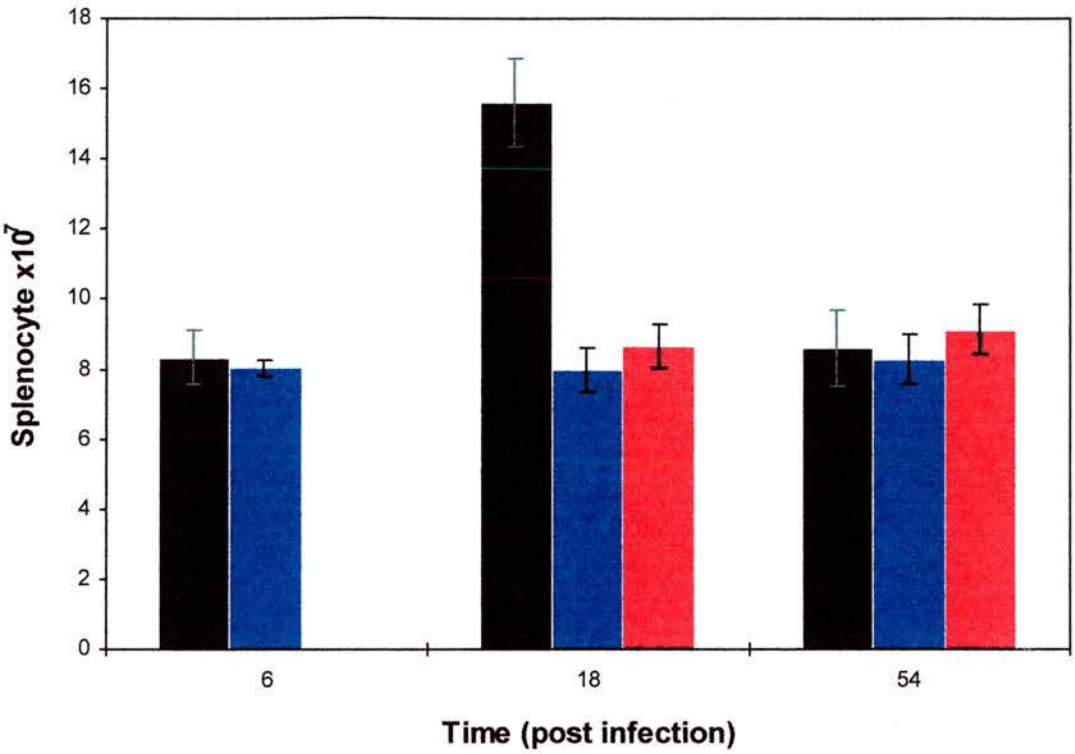


Figure 4.3.4 The number of splenocytes observed in mice infected intra-nasally with $4x10^5$ pfu MHV-68. The mice were either left untreated (black), prophylactically 4'-s-EtdU treated and not withdrawn (blue) or withdrawn from treatment day 12 post infection (red). Three mice from the untreated group were sampled at day 6, 4 mice at day 18 and 5 mice at day 31 post infection. Three mice from the prophylactically treated (and not withdrawn) group were sampled at day 6 and 4 mice at days 18 and 54 post infection. Four mice from the treatment withdrawn (at day 12 post infection) group were sampled at day 18 and 5 mice at day 54 post infection. The values are represented by arithmetic means and the error bars represent the standard deviation.

Figure 4.3.5

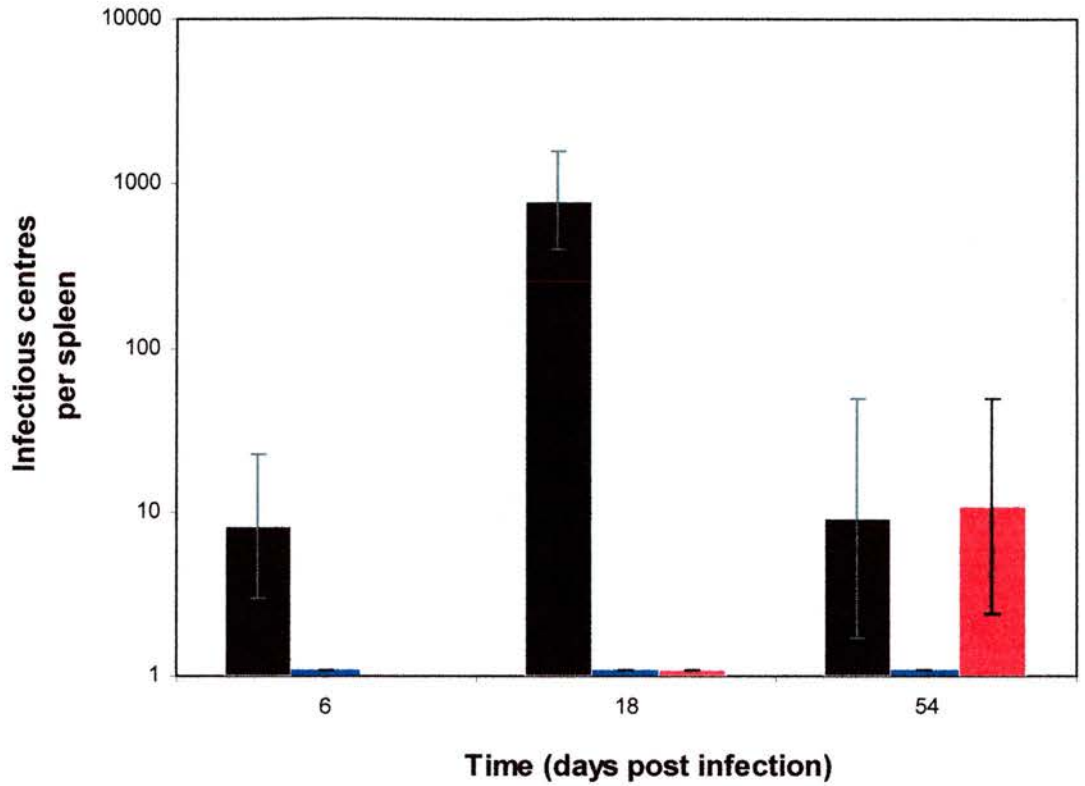


Figure 4.3.5 The latent virus titres (as measured by infectious centre assay) detected in the spleens of mice infected with $4x10^5$ pfu MHV-68, via the intra-nasal route. The mice were either left untreated (black), prophylactically 4'-s-EtdU treated and not withdrawn (blue) or withdrawn from treatment day 12 post infection (red). Three mice from the untreated group were sampled at day 6, 4 mice at day 18 and 5 mice at day 31 post infection. Three mice from the prophylactically treated (and not withdrawn) group were sampled at day 6 and 4 mice at days 18 and 54 post infection. Four mice from the treatment withdrawn (at day 12 post infection) group were sampled at day 18 and 5 mice at day 54 post infection. The values are represented by geometric means and the error bars represent the standard deviation. The assay limit of detection was 5 infectious centres per spleen.

MHV-68 specific 40 cycle PCR was carried out on DNA samples extracted from the splenocytes of the mice sampled at day 54 post infection. MHV-68 specific PCR bands of 500bp was produced by 3/4 untreated infected mice tested and with all mice withdrawn from treatment at day 12 post infection (4/4). However, a MHV-68 specific PCR band was not produced by the infected mice which were either not withdrawn from treatment (0/4) or withdrawn on day 51 post infection (0/4) (*see figure 4.3.6a*). A further 25 rounds of nested PCR was carried out on the DNA samples from the infected mice which were either not withdrawn from treatment, withdrawn from treatment on days 51 post infection or left untreated. All the untreated mice splenic DNA samples (4/4) gave rise to a MHV-68 specific nest PCR band at 400bp. However, a MHV-68 specific PCR band was not produced by any of the mice that were either not withdrawn from treatment (0/4) or withdrawn from treatment on day 51 post infection (0/4) (*see figure 4.3.6b*).

4.3.3 **The prophylactic 4'-s-EtdU treatment of BALB/c mice, infected via the intra-peritoneal route.**

MHV-68 infections initiated in the peritoneal cavity, give rise to a more systemic infection than when initiated in the lungs. Viral latency can be detected earlier in the spleens of mice infected via the intra-peritoneal route and the associated splenomegaly is more severe, than with mice infected via the intra-nasal route. An experiment was therefore set up to compare and contrast the effects of prophylactic 4'-s-EtdU treatment on mice infected via the intra-peritoneal route. An experiment to determine whether productive virus replication is required merely as a means of crossing the lung epithelium or whether it plays a more fundamental role in the establishment of viral latency in splenic B-lymphocytes.

Thirteen BALB/c female mice, aged 3 to 4 weeks were randomly selected from a batch of 22 age and sex matched animals and were put on drinking water containing 0.3mg/ml 4'-s-EtdU. The remaining 9 animals remained on normal drinking water. After 2 days of treatment, the mice on 4'-s-EtdU drinking water and the normal

Figure 4.3.6

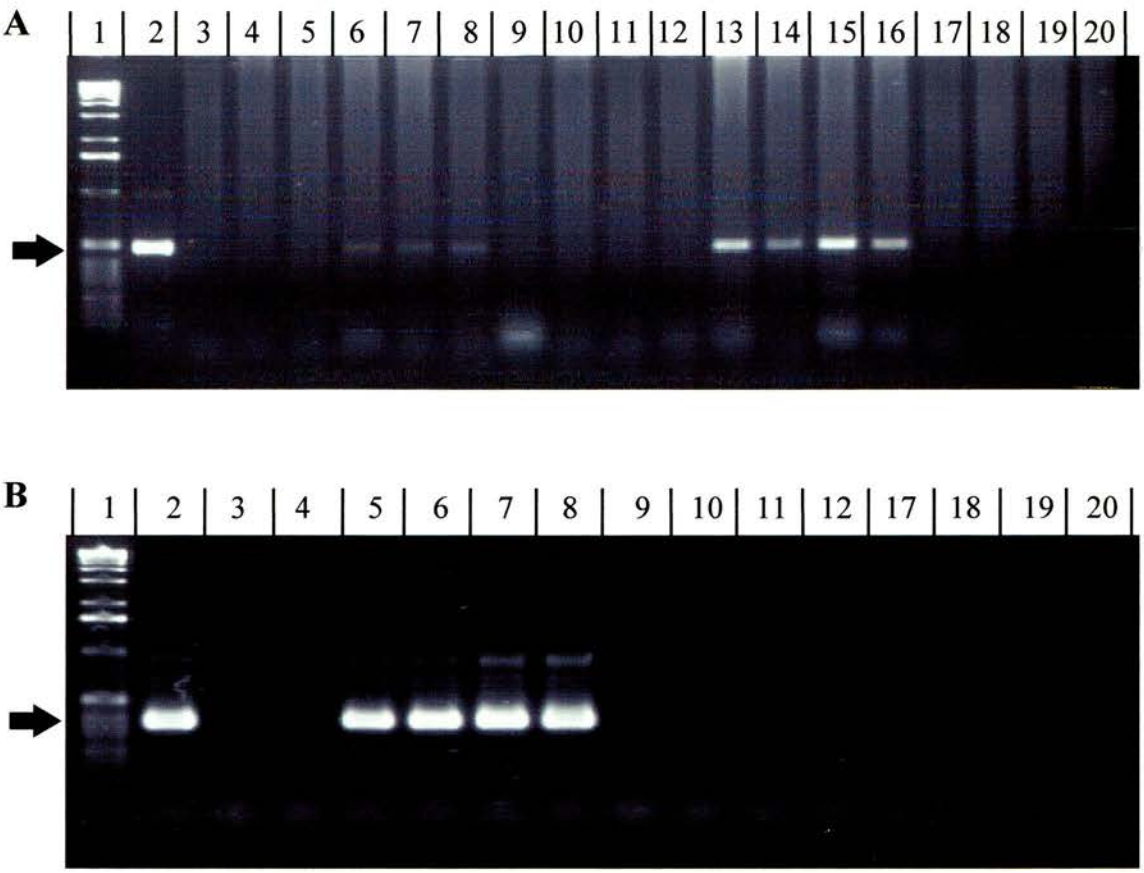


Figure 4.3.6 The long term effects of prophylactic 4'-s-EtdU treatment on the on viral persistence in the spleen of MHV-68 mice infected mice, as determined by PCR. First round 40 cycle MHV-68 specific PCR was carried out on 500ng of DNA extracted from splenocytes of BALB/c mice, infected intra-nasally with $4x10^5$ pfu MHV-68 (A). A second round of 25 cycle MHV-68 specific nested PCR was also carried out on 10μl of the first round PCR products (B). The infected mice were either left untreated (lanes 5 to 8), prophylactically treated with 4'-s-EtdU and not withdrawn (lanes 9 to 12), withdrawn from treatment at 12 days post infection (lanes 13 to 16) or withdrawn from treatment at 51 days post infection (lanes 17 to 20). All mice were sampled on day 54 post infection. Lane 2 represents the PCR positive control and was carried out on 200ng of DNA extracted from MHV-68 persistently infected NS0 cells. Lanes 3 and 4 represent PCR negative controls and were carried out on 500ng of DNA extracted from splenocytes of uninfected BALB/c mice. The products were visualised by 2% agarose gel electrophoresis and ethidium bromide staining, viewed under UV. Product size was determined by comparison to 1kbp Ladder (lane 1). For further information see Table 4.7.

Table 4.7 **The summarised results in *figure 4.3.6* (the first round and nested MHV-68 specific PCR, carried out on splenic DNA extracted from both 4'-s-EtdU treated and untreated MHV-68 infected mice).**

Lane Number	Sample Description	Mouse Number	Route of MHV-68 Inoculation	[1] Day Sampled	1st Round PCR Results	Nested PCR Result
1	1KB Ladder	-	-	-	-	-
2	[2] Positive Control	-	-	-	+ve	+ve
3	Uninfected spleen	1	-	-	-ve	-ve
4	Uninfected spleen	2	-	-	-ve	-ve
5	Group 1 spleen	1	Intra-nasal	54	-ve	+ve
6	Group 1 spleen	2	Intra-nasal	54	+ve	+ve
7	Group 1 spleen	3	Intra-nasal	54	+ve	+ve
8	Group 1 spleen	4	Intra-nasal	54	+ve	+ve
9	Group 2A spleen	1	Intra-nasal	54	-ve	-ve
10	Group 2A spleen	2	Intra-nasal	54	-ve	-ve
11	Group 2A spleen	3	Intra-nasal	54	-ve	-ve
12	Group 2A spleen	4	Intra-nasal	54	-ve	-ve
13	Group 2B spleen	1	Intra-nasal	54	+ve	ND
14	Group 2B spleen	2	Intra-nasal	54	+ve	ND
15	Group 2B spleen	3	Intra-nasal	54	+ve	ND
16	Group 2B spleen	4	Intra-nasal	54	+ve	ND
17	Group 2C spleen	1	Intra-nasal	54	-ve	-ve
18	Group 2C spleen	2	Intra-nasal	54	-ve	-ve
19	Group 2C spleen	3	Intra-nasal	54	-ve	-ve
20	Group 2C spleen	4	Intra-nasal	54	-ve	-ve

Key: **-** ~ Not applicable, **ND** ~ sample not tested, **-ve** ~ negative, **+ve** ~ positive, [1] ~ Time when mice were killed (days post infection), [2] ~ DNA extracted from MHV-68 infected NS0 cells. The **Group 1** mice were left untreated, the **Group 2** mice were treated prophylactically with 4'-s-EtdU and either not withdrawn (**A**), withdrawn from treatment on day 12 post infection (**B**) or withdrawn from treatment on day 51 post infection (**C**).

drinking water were infected via the intra-peritoneal route, with $4x10^5$ pfu MHV-68. At day 12 post infection, 4 of the mice put onto 4'-s-EtdU drinking water were withdrawn from treatment. The remaining prophylactically treated mice remained on the 4'-s-EtdU drinking water for the duration of the experiment.

4.3.4 **The effect of prophylactic 4'-s-EtdU treatment on the dissemination of MHV-68 to the lungs of mice following intra-peritoneal infection.**

The mice infected via the intra-peritoneal route were sampled at day 10 and 24 post infection and lungs and spleens removed for analysis. No infectious virus could be detected in the lungs of any of the mice infected intra-peritoneally at either days 10 or 24 post infection, by direct plaque assay. However, by co-cultivation assay, virus was reactivated from the lungs of all the untreated mice tested (4/4). Virus could not be reactivated from any of the lung tissue samples derived from the prophylactically treated (0/4) mice and only 1/4 mice withdrawn from treatment, sampled at day 24 post infection (*see figure 4.4.1*).

MHV-68 specific PCR was carried out on DNA extracted from lungs derived from the mice sampled at day 24 post infection. The DNA extracted from the lungs of all the untreated mice (5/5) gave rise to a MHV-68 specific band after 40 cycles of PCR, as did 1/4 mice withdrawn from the 4'-s-EtdU treatment (the same mouse which was virus positive by lung co-cultivation and splenic infectious centre assay). However, a MHV-68 specific band at 500bp, was not produced from any of the lung DNA samples derived from the 4'-s-EtdU treated mice that were not withdrawn from treatment (*see figure 4.2.7b*). After a further 25 cycles of nested PCR, only 2/4 and 1/4 mice prophylactically treated and either withdrawn or not withdrawn from treatment, gave rise to a MHV-68 specific band, at 400bp, respectively (*see figure 4.2.7c*).

Figure 4.4.1

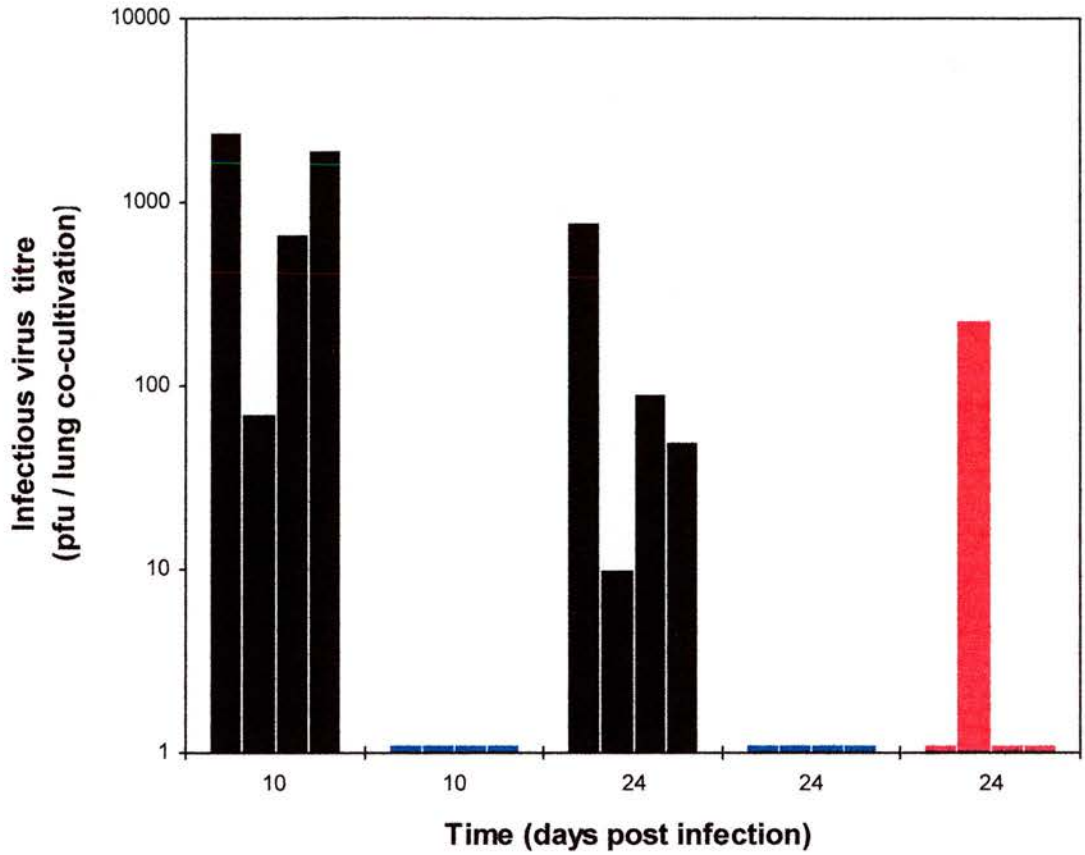


Figure 4.4.1 The levels of infectious virus reactivated (by co-cultivation assay) from the lung tissue of mice infected intra-peritoneally with $4x10^5$ pfu MHV-68. The mice were either left untreated (black), prophylactically 4'-s-EtdU treated and not withdrawn (blue) or withdrawn from treatment day 12 post infection (red). Four mice per group were sampled at both days 10 and 21 post infection. The assay limit of detection was 5 pfu per co-cultivation.

4.3.5 **The effect of prophylactic 4'-s-EtdU treatment on splenomegaly induction, following intra-peritoneal infection.**

A pronounced splenomegaly occurred in the untreated mice infected via the intra-peritoneal route. The numbers of splenocytes observed, at both day 10 and day 24 days post infection, was approximately double the number which would normally be expected in uninfected mice (*see figure 4.4.2*). A splenomegaly was not observed in either the prophylactically treated mice which were not withdrawn from treatment, at both days 10 and 24 post infection, or the mice withdrawn from treatment, sampled at day 24 post infection. There were significantly more splenocytes observed in the untreated mice than the mice withdrawn from the prophylactic treatment as well as the non-withdrawn mice, sampled at both days 10 and 24 post infection (P=0.046, 0.003 and 0.021 respectively, by student T test). There was no significant difference in the number of splenocytes observed in the 4'-s-EtdU treated mice, withdrawn and non-withdrawn, sampled at day 24 post infection (P=0.94).

4.3.6 **The effect of prophylactic 4'-s-EtdU treatment on the dissemination of MHV-68 to the spleen, following intra-peritoneal infection**

All the untreated mice gave rise to infectious centres at both days 10 and 24 post infection, averaging $8.9x10^3$ and $1.3x10^2$ infectious centres per spleen, respectively. Conversely, only 1/4 prophylactically 4'-s-EtdU treated mice, at day 10 post infection and 0/4 and 1/4 mice withdrawn and non-withdrawn from treatment, respectively, had a detectable infectious centre titre (*see figure 4.4.3*).

The DNA extracted from the splenocytes of all 5 untreated mice sampled at day 24 post infection, gave rise to a MHV-68 specific band at 500bp, after 40 cycles of PCR, as did 1/ 4 mice withdrawn from the 4'-s-EtdU treatment (the same mouse which was virus positive by lung co-cultivation assay and splenic infectious centre assay). However, MHV-68 specific bands was not produced by any of the DNA samples derived from splenocytes of the non-withdrawn 4'-s-EtdU treated mice (*see*

Figure 4.4.2

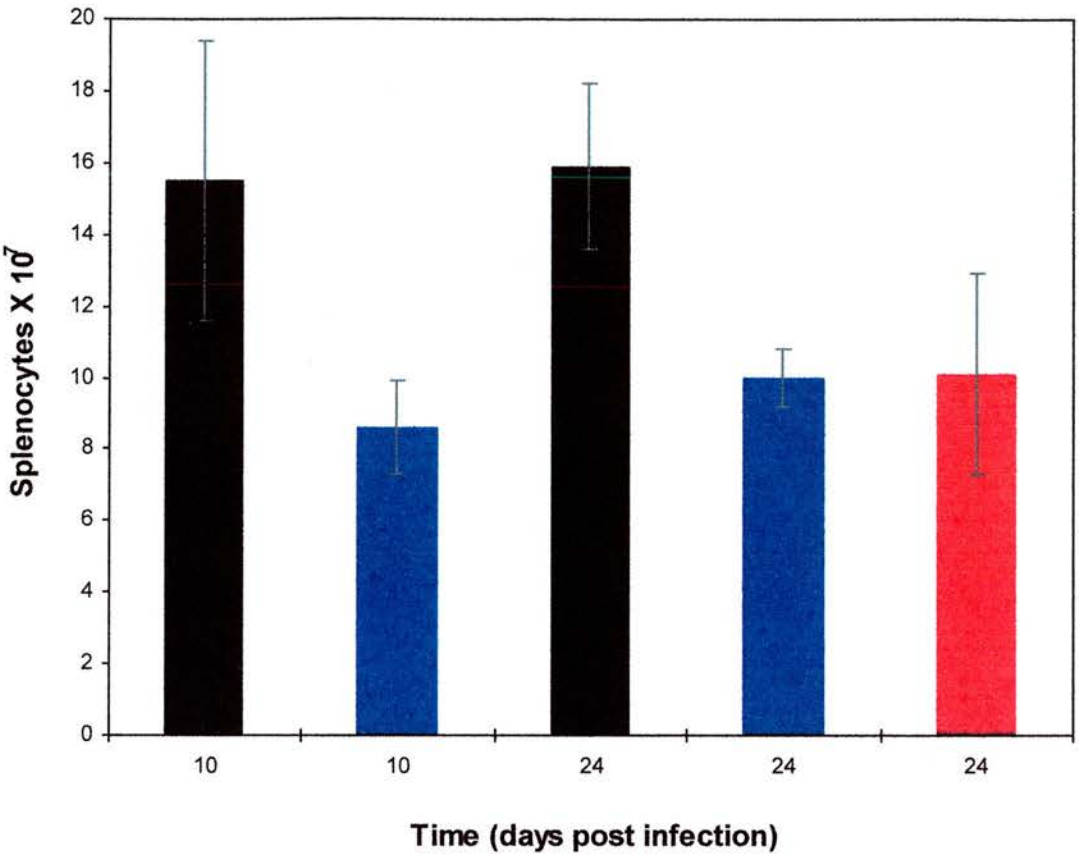


Figure 4.4.2 The number of splenocytes observed in mice infected (ip) with $4x10^5$ pfu MHV-68. The mice were either left untreated (black), prophylactically 4'-s-EtdU treated and not withdrawn (blue) or withdrawn from treatment day 12 post infection (red). Four mice per group were sampled at both days 10 and 21 post infection. The values are represented by arithmetic means and the error bars represent the standard deviation.

Figure 4.4.3

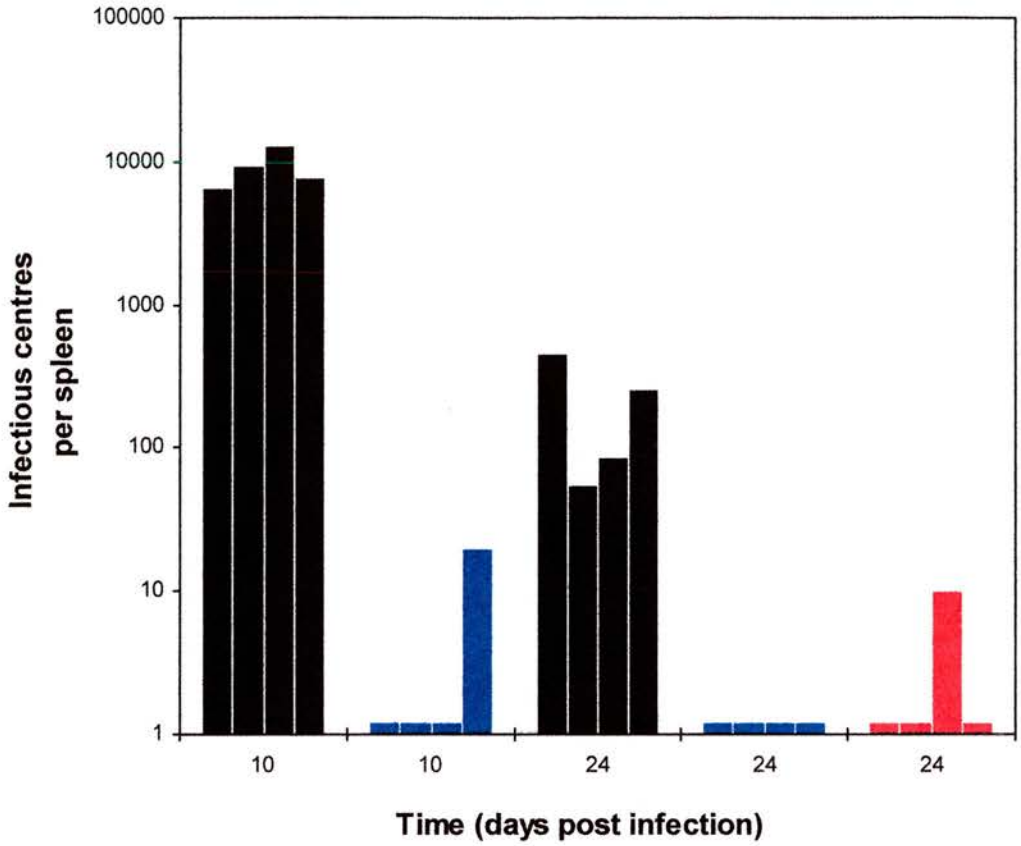


Figure 4.4.3 The latent virus titres (as measured by infectious centre assay) detected in the spleens of mice infected with $4x10^5$ pfu MHV-68 via the intra-peritoneal route. The mice were either left untreated (black), prophylactically 4'-s-EtdU treated and not withdrawn (blue) or withdrawn from treatment day 12 post infection (red). Four mice per group were sampled at both days 10 and 21 post infection. The assay limit of detection was 5 infectious centres per mouse.

figure 4.2.5). After a further 25 cycles of nested PCR, all the mice withdrawn (4/4) and 2/4 mice not withdrawn from the 4'-s-EtdU treatment, gave rise to MHV-68 specific bands, at 400bp (*see figure 4.2.6*).

4.3.7 The potency of 4'-s-EtdU at inhibiting the productive replication of MHV-68, *in vivo*

The earlier *in vitro* studies provided clear evidence of the ability of 4'-s-EtdU to inhibit the productive replication of MHV-68 in infected tissue culture cell lines. However *in vitro* anti-viral activity does not always translate well to the *in vivo* situation. The ability of 4'-s-EtdU to significantly inhibit the productive replication of MHV-68 was demonstrated by the fact that treatment from day 3 post infection, significantly shortened the acute lung infection, following intra-nasal infection. No infectious virus was detectable in the lungs after 4 days of treatment, a time point at which infectious virus was readily detected in all the mock-treated mice. The absence of infectious virus was unlikely to have been due to 4'-s-EtdU carryover from the lung tissue, since earlier studies have shown that 4'-s-EtdU treatment (1mg/mouse/day from day 3 post infection) does not prevent the plaquing of MHV-68 present in the lungs of mice (Barnes *et al*, unpublished data). At day 12 post infection, treatment continued via 4'-s-EtdU supplemented drinking water. The administration of 4'-s-EtdU via drinking water was an effective means of treatment, since no infectious virus was detected in the lungs of the mice transferred to 4'-s-EtdU supplemented drinking water (at the point of infection), by day 3 post infection.

The evidence from the experimental time course carried out, where treatment started at day 3 post infection, did not determine the exact extent of the levels of viral inhibition exerted by the 4'-s-EtdU. However the evidence provided by the 2 time courses following intra-nasally infected, prophylactically 4'-s-EtdU treated mice, strongly suggested that the oral administration (via drinking water at 0.3mg/ml) of 4'-s-EtdU, completely inhibited the productive replication of MHV-68. The evidence in part, comes from the fact that no infectious virus could be detected in the lungs of the intra nasally infected mice at days 3, 6 and 7 post infection. The

untreated mice had infectious virus titres of $1.6x10^4$, $1.8x10^5$ and $7x10^4$ pfu per lung respectively at these time points. As previous discussed, it is unlikely that the absence of detectable pfu in the lungs of the prophylactically treated mice was due to the presence of 4'-s-EtdU (within the lung tissue) carrying over into the *in vitro* assay and prevented virus plaquing.

Supporting this evidence is the fact that for up to 54 days post infection, the prophylactically treated mice failed to produce antibodies specific to MHV-68 late antigens. Failure of the treated mice to produce antibodies anti-MHV-68 specific antibodies occurred despite the presence of MHV-68 genomic DNA in the lung tissue. Had the viral DNA (or undetected virus, potentially present at other sites within the mouse) represented virus that underwent a very low level of productive / chronic viral replication in the mice, the mice would have been expected to have mounted a detectable humoral response to the viral antigens expressed. A pronounced humoral response was readily detectable in the untreated positive control mice (by day 18 post infection) and the mice withdrawn from treatment at day 12 post infection mice.

4.3.8 **The role of productive virus replication in the establishment and maintenance of viral latency in the spleen**

As shown by earlier work, mice treated with 4'-s-EtdU from day 3 post infection, experienced a short term but significant delay in the appearance of splenic infectious centres. At day 7 post infection, the spleens from all the untreated mice (3/3), but known of the treated mice (0/4) were infectious centre positive. The infectious centre titre in the treated mice was also significantly lower than in the untreated mice, at day 12 post infection, which was the peak infectious centre time point for the untreated mice. However, by day 26 post infection, there was no significant difference between the infectious centre titres observed in the mice treated and not withdrawn, treated and withdrawn and those left untreated. This remained the case throughout the rest of the experiment. Clearly the suppression of viral latency in

splenocytes, seen at the early time points, did not have a significant effect on the establishment or maintenance of long-term viral latency. The reason for the delay in the appearance of splenic infectious centre was presumably due to the decreased severity of the acute lung infection and hence an initial reduction in the number of infected circulating B-lymphocytes. This data is therefore in agreement with other studies carried out to determine the effect of ACV treatment on EBV infected patients (Zutter *et al*, 1988, Yao *et al*, 1989a & b Luxton *et al*, 1993). In these studies the anti-viral treatment failed to significantly affect the levels of viral B-cell latency in the patients. Similarly, ACV, VACV or FCV *in vivo* treatment initiated after infection, despite being highly potent inhibitors of HSV replication, do not prevent the establishment and maintenance of latency in neurones (Field *et al*, 1981 & 1997 and Thackray *et al*, 1996a).

The data from the prophylactic 4'-s-EtdU treatment experiments strongly suggests that treatment of mice with 4'-s-EtdU, at the level used in this experiment (from day 3 post infection) completely inhibits productive viral replication of MHV-68. It is reasonable then to assume that the 4'-s-EtdU treatment inhibited all productive viral replication for the duration of the treatment. Selective outgrowth of 4'-s-EtdU resistant viral variants did not occur during treatment, since virus reactivated from the lungs and splenocytes of treated and treatment withdrawn mice failed to show any loss of sensitivity to 4'-s-EtdU. The data from this study is therefore consistent with the hypothesis that the long-term maintenance of viral latency in B-lymphocytes does not have a requirement for productive or chronic viral replication (up to day 61 post infection), either within the lymphocyte compartment or at other sites, such as the lungs. It also implies that the long term treatment of infected mice does not affect the ability to detect latently infected splenocytes by infectious centre assay (as was the case for infected NS0 cell lines - see chapter 3).

Virus failed to spread to the spleen in the prophylactic 4'-s-EtdU treated mice which were not withdrawn from treatment, as determined by nested PCR and by splenocyte co-cultivation assay. In immunologically normal MHV-68 infected mice, the spleen,

being the largest lymphoid organ and a major site for lymphocyte trafficking, normally possesses the highest density of latently infected B-lymphocytes, within the lymphatic (or blood) system (Sunil-Chandra *et al*, 1992). Despite this, viral genomic DNA remained undetectable by nested PCR in all prophylactic 4'-s-EtdU treated mice not withdrawn from treatment, up to 54 days post infection (0/11 mice over both time courses). Virus genomic DNA was detectable (by either first round or nested PCR) in all the untreated, infected mice tested in both the prophylactic 4'-s-EtdU treatment experiments (11/11). These results clearly demonstrate the need for productive replication (probably at the sight of the acute infection) to establish a latent infection of circulating B-lymphocytes, following intra-nasal infection.

The evidence provided by the prophylactic 4'-s-EtdU treatment of mice infected via the intra-peritoneal route, suggests other sites of viral persistence may exist in the mouse, other than the lungs and lymphoid cells. Although the possibility can not be ruled out that persistent virus may reside at sites other than the lungs, following intra-nasally infection, it is hard to conceive how the virus could pass from the lungs to a different site in the mouse without coming into contact with circulating B-lymphocytes (*in vitro* cell lines including those of B-cell origin can be infected in the presence of high doses 4'-s-EtdU - see chapter 3). After the 4'-s-EtdU treatment was withdrawn, the virus disseminated to the spleen. However, dissemination occurred gradually, over a period of weeks. Virus could be reactivated from the lungs of all the mice withdrawn from treatment on day 12 post infection, by day 21 post infection (9 days after withdrawal). However, at day 21 post infection, virus could not be reactivated from the splenocytes of the same mice and only 1/4 mice had virus genomic DNA present in the splenocyte DNA preparations, as determined by nested PCR. However, all the mice withdrawn from the prophylactic 4'-s-EtdU treatment eventually established a splenic infection. All the mice tested 19 days after treatment withdrawal (3/3) were virus positive by nested PCR and (2/3) by infectious centre assay. However, even after 19 days of treatment withdrawal, the levels of viral DNA present in the spleen appeared to be lower than in the untreated mice. MHV-68 DNA could not be detected in the treatment withdrawn mice (0/3) by first

round PCR, when it could in 2/3 untreated mice. By day 54 post infection (42 days post treatment withdrawal) levels of viral latency in the spleens of the mice withdrawn from treatment had risen to equivalent (if not higher) titres to those observed in the untreated mice. Virus could be reactivated from 3/5 mice withdrawn from treatment on day 12 post infection, whereas virus was only reactivated from 2/5 of the untreated mice. Also, all the mice tested (4/4) produced bright virus specific bands by first round PCR, whereas only 3/4 untreated mice produced virus specific bands, all of which were of low intensity.

4.3.9 **The role of productive virus replication in the induction of splenomegaly**

Despite the obvious *in vivo* potency of 4'-s-EtdU and the suppression of latent virus titres associated with the spleen at early time points, the treatment failed to prevent the viral induction of splenomegaly in mice treated from day 3 post infection. The splenocyte counts in the treated mice failed to show any significant differences from those of the untreated mice, at any of the time points studied (days 7, 12, 26, 38, 48 and 61). Therefore is it seems likely that productive replication (or late gene expression) does not play a role in the induction of splenomegaly or lymphoproliferation. Similar results have been seen with the ACV treatment of patients with EBV associated infectious mononucleosis and LPD. Treatment reduces the viral shed from the oro-pharynx, but failed to impact on the course of the disease, or the levels of viral latency in B-lymphocytes (Zutter *et al*, 1988 and Yao *et al*, 1989a). However, unlike with the EBV studies, the anti-viral treatment, in this study, started at least 4 days prior to the proliferation of lymphocytes.

The splenomegaly normally seen in mice infected with MHV-68 has been shown to depend on the presence of infected B-lymphocytes and CD4 positive T-cells (Usherwood *et al*, 1995 & 1996a). None of the prophylactically 4'-s-EtdU treated mice, infected via the intra nasal route, developed splenomegaly. This was presumably due to the lack of viral dissemination to the spleens of the infected mice. However, splenomegaly was not observed in the mice withdrawn from the 4'-s-EtdU

treatment in which, over time, viral latency was established in splenocytes. Moreover, by nested PCR it was shown that the virus reached the spleen of a number of prophylactically treated mice infected intra peritoneally, albeit at a low level. Splenomegaly did not occur in the prophylactically treated mice infected via the intra peritoneal route. This data suggests that splenomegaly is induced by a sudden influx of latently infected B-lymphocytes and does not occur if latently infected B-cells accumulate in the spleen gradually and at very low levels.

4.4.1 **The role of productive virus replication in the establishment and maintenance of viral persistence in the lung**

The lung has previously been described as a site of viral persistence, both in normal inbred mice (Sunil-Chandra - PhD thesis) and in μMT and class II knockout mice (Usherwood *et al*, 1995 and Cardin *et al*, 1996). The evidence from the μMT study demonstrated that viral persistence in the lung could be established and maintained in the absence of a latent population of circulating B-cells. The evidence from the class II knock out mouse study, demonstrated clear evidence of a chronic infection in the lung at delayed late time points.

After the acute infection, virus could not be detected by direct plaque assay, in the lungs of any of the mice. However, virus could be reactivated from the lung tissue of the untreated mice and the mice withdrawn from treatment. It is at present not known which cells in the lung tissue harbour the persistent virus. A possible candidate would be latently infected circulating B-lymphocytes, since the lungs were not perfused prior to dissection. However, this is unlikely since 4'-s-EtdU treatment prevented the reactivation of infectious virus from the lungs but not from splenocytes (both by co-cultivation assay) from the same animals. Virus was reactivated from the lungs of all the mock-treated and mice withdrawn from treatment (9/9 and 10/10 respectively), but generally not from the lungs of the mice that were treated but not withdrawn (1/12). Despite this, there was no significant difference in the ability to reactivate virus from splenocytes derived from the mice from either the mock-

treated, treated and withdrawn or treated not withdrawn mice (7/8, 11/12 and 10/12 respectively). Therefore the fact that virus could not be reactivated from the lungs of the treated and not withdrawn mice, could not be attributed to a lower number of latently infected B-lymphocytes, detected by infectious centre assay. It would therefore appear that the persistent virus present in the lung tissue of the intra-nasally infected mice was independent of latently infected B-cells.

The fact that the 4'-s-EtdU treatment appeared to have an inhibitory effect on the persistent virus infection of the lungs, implies viral persistence at this site has a chronic aspect. This result is highly reminiscent of chronic EBV associated syndromes, such as OHL (Renick *et al*, 1988 and Thomas *et al*, 1991) and cryptogenic fibrosing alveolitis (Egan *et al*, 1995), both of which have been associated with the chronic replication of EBV oro-respiratory tract.

The evidence from prophylactic 4'-s-EtdU treatment experiments also supports the evidence from the 4'-s-EtdU treatment from day 3 post infection experiment, that there is normally a chronic element to the viral persistence in the lungs, in immunologically normal inbred mice. Virus could be reactivated from the lung tissue of the untreated mice and the treated mice once withdrawn, at the later time points day 21 and 31 post infection. However, as was shown with the mice treated from day 3 post infection, virus could be reactivated far less efficiently from the lung tissue of the mice not withdrawn from treatment and only at the earliest time point (day 12 post infection). The level of viral DNA was also lower in the mice not withdrawn from treatment than in the lungs of the untreated and treatment withdrawn mice, tested at day 21, 31 and day 54 post infection. Virus genomic DNA could be readily detected by first round PCR in the lungs of virtually all the untreated and treatment withdrawn mice and did not requiring a second round of nested PCR. Virus could not be detected in the lung tissue of the continually treated mice, by first round PCR. However, MHV-68 genomic DNA could be detected in the lung tissue of all the continually treated mice by nested PCR. This result suggests that the reason why virus could not be reactivated from the lung tissue of continually treated

mice, (i.e. the prophylactically treated mice and in all likelihood the mice treated from day 3 post infection) was due to a decrease in viral load and not due to elimination of persistent virus from this site. The increased viral load observed in the lungs of the untreated mice and mice withdrawn from treatment, is strong evidence of a low level chronic infection occurring in the lungs since 4'-s-EtdU should not affect the viral genome copy number in a 'tightly' latent infection.

However, the evidence from the prophylactic 4'-s-EtdU treatment experiments also strongly suggests that viral persistence in the lungs is not dependant on chronic / productive virus replication or the presence of latently infected circulating B-lymphocytes for low level establishment and maintenance. Virus DNA could be detected in all the prophylactically treated mice following intra-nasal infection, up to day 54 of continuous treatment, by nested PCR. Therefore viral persistence, at the DNA level, could not have been reliant on productive and hence chronic virus replication in the lungs, or by reseeding, from latently infected circulating B-cells. The viral persistence observed in the lungs of the prophylactically treated mice is reminiscent of the viral persistence seen in 4'-s-EtdU treated infected MGC-7 cells and must either represent a form of latent infection or a form of herpesvirus persistence hitherto undefined. It is unlikely that that the persistent virus seen in the lungs was due to cell free virus from the inoculum, because MHV-68 is a highly cell associated virus which, at room temperature or higher, rapidly loses viability in a cell free environment (*unpublished observations*).

4.4.2 **The viability of persistent MHV-68 in the lungs of the prophylactically 4'-s-EtdU mice following intra-nasal infection**

Although the virus present in the lungs of the prophylactically 4'-s-EtdU treated mice, up to 12 days post infection, was shown to be viable (by co-cultivation assay on withdrawal of the treatment), the virus genomic DNA there after, was not formally shown to be viable. This was due to the unexpected development of 4'-s-EtdU associated renal toxicity in the experiment designed to determine the long-term

effects of prophylactic 4'-s-EtdU treatment. Twelve days is sufficient time for supercoiling, methylation and conversion to chromatin to occur within a cell. However, on withdrawal of the treatment, productive replication occurred, denoted by the sera conversion of the mice to MHV-68 late antigens, and the virus dissemination to the spleen (as determined by PCR, infectious centre and splenocyte co-cultivation assay). It is therefore considered likely that the persistent viral genomic DNA, observed in the prophylactically treated mice after day 12 post infection, represented potentially viable MHV-68 genomes. Although 12 days is a relatively short period of time compared to 54 days, MHV-68 can exist in both NS0 and MGC7 cell lines, in the presence of inhibiting doses of 4'-s-EtdU, for in excess of 60 days without the loss of virus viability (*see Chapter 3*).

The evidence from 1/ the progressive seeding of virus to the spleen after withdrawal of treatment, which was 2/ accompanied by the production of antibodies specific to MHV-68 late antigens and 3/ preceded by an increase in the levels of viral DNA in the lungs and an increase in the ability to reactivate virus from the lungs, is concurrent with the hypothesis that MHV-68, in the absence of productive replication, is held in the lung tissue, following intra-nasal infection and can not disseminate to the lymphoid compartments of the mouse. Only on removal of the productive virus replication block, can the virus then seed to B-lymphocytes and hence to the spleen.

4.4.3 **The role of productive virus replication in the dissemination of MHV-68 following infection initiating in the peritoneum**

Infection of mice with MHV-68 via the intra-peritoneal route normally results in a greater systemic infection than infection via the intra-nasal route. Despite this the prophylactic 4'-s-EtdU treatment still greatly hampered the establishment of splenic latency. Only 1/8 prophylactically treated mice had an assayable splenic infectious centre titre and only 2/4 mice tested, were virus positive by nested PCR. In contrast, all the untreated mice (9/9) had an assayable infectious centre titre and virus DNA

could be detected in the splenocytes of all the mice tested, by first round PCR, as opposed to nested PCR. Even after the treatment had been withdrawn for 12 days, only 1/4 mice were infectious centre and first round PCR positive, although all four mice were virus DNA positive by nested PCR. This suggests that productive replication is essential for the efficient establishment of viral latency in B-lymphocytes, when infected via the intra-peritoneal route. A possible explanation for this is that the inflammatory response made at the sites of acute or chronic virus infection is necessary for the recruitment of B-cells to the site and hence the efficient infection there of.

Virus could be detected in the lungs of all the untreated mice tested, by both co-cultivation assay (9/9) and by first round PCR (5/5), demonstrating the natural tropism of MHV-68 for this site. However, the lungs of only 1/4 mice prophylactically 4'-s-EtdU treated and not withdrawn, and 2/4 mice withdrawn from treatment on day 12 post infection mice, were MHV-68 DNA positive, by nested PCR. The treated and not withdrawn mice, which had MHV-68 DNA present in the lung, also contained detectable MHV-68 DNA in the spleen. This indicates that the initial infecting virus inoculum achieved better systemic spread than with the other mice of the same group. No mouse of any group had detectable virus DNA in the lungs in the absence of virus in the spleen. However, 2/4 mice not withdrawn from treatment, did not have detectable virus in either the spleen or the lungs. Despite the low numbers of mice sampled, these results suggest there may well be a third site of viral persistence, other than the lungs or circulating B-lymphocytes. Further research will be necessary to identify the alternative site of persistence open to gammaherpesviruses.

Chapter 5: The potential role of productive viral replication in the oncogenesis of MHV-68.

5.1.1 Summary

A number of experiments were carried out in an attempt to generate a reliable model for MHV-68 LPD development in mice. This was done with the overall aim to determine the potential therapeutic value of 4'-s-EtdU in the treatment of gammaherpesvirus associated LPD. Also to determine the value of 4'-s-EtdU as a tool for the study of LPD in mice with impaired immune systems. MHV-68 infections are accompanied with a greater degree of lytic virus replication than has been described for other gammaherpesviruses, potentially leading to severe complications in immunocompromised hosts. C57BL/6 mice were immunosuppressed with CsA for 2 week, during the post acute MHV-68 infection. The mice were kept under observation for 2 years, to determine whether MHV-68 associated tumour development occurred as readily in mice of the C57BL/6 background, as has been reported for mice of the BALB/c background. Despite a significant increase in viral latency in splenocytes, during the CsA treatment, the latent virus titres returned to normal after withdrawal of treatment. No lymphomas or lymphoproliferative disease were observed in any of the mice. SCID mice were also infected with MHV-68 following adoptive transfer with syngeneic splenocytes. The SCID mice could be protected from the otherwise lethal MHV-68 infection by both 4'-s-EtdU supplemented drinking water or CD8 depleted or non-depleted splenocyte adoptive transfers, from both infected and uninfected donor mice. Latent virus could be detected in the spleens of the splenocyte adoptive transfer recipient, MHV-68 infected SCID mice, at early time points (up to day 21 post adoptive transfer) but only rarely in the later time point mice (day 36 to 60 post infection mice). None of the mice developed splenic lymphomas or lymphoproliferative disease, as defined by increased in latent virus load or increase in splenocyte count. The study failed to demonstrate a role for CD8 T-cells in either the control of latent virus titres or disease development.

5.1.2 The role of CsA in the development of EBV associated lymphoproliferative disease

CsA can suppress both T and B cell proliferation, by antagonising the calcium ion signal pathways. Calcium signalling is essential for IL-2 synthesis leading to T-cell activation and proliferation. As with other immunosuppressive regimes CsA treatment is associated with the development of EBV associated LPD (see introduction), making it a good *in vivo* reagent to study herpesvirus infection under immunosuppressive conditions (Jayasuriya *et al*, 1983 and Veronese *et al*, 1992). CsA has been used in many animal model systems as an immunosuppressant or tolerance inducers (For general review see - Thomson *et al*, 1989). CsA is also used *in vitro*, to promote the out-growth of, EBV-immortalised, LCLs from PBLs donated by EBV sera positive, individuals (Rickinson *et al*, 1986).

5.1.3 The role of CsA in MHV-68 associated lymphoproliferative disease

Previous studies carried out on the MHV-68 long-term infection of inbred mice strains (predominantly BALB/c), have shown that 9% of mice develop LPD over a period of 9 month to 3 years post infection. Nine of the twenty LPD (45%) cases, were judged to be high grade lymphomas. The lymphomas were comprised of both light chain restricted B-cells and infiltrating T-cells. However, 60% of mice, treated early on in the infection with CsA (50mg/kg) and allowed to fully recover, went on to develop LPD over a 12 month period. Over the same period of time 20% of the untreated (MHV-68 infected) mice developed LPD. No lymphomas were seen in the 300 uninfected control mice, however, no uninfected mice were CsA treated.

Inbred mice strains lacking specific elements of the immune system would be ideal for the study of the *in vivo* oncogenic potential of MHV-68. However, it is hard to

study the tumour forming abilities of MHV-68 in mice with a significantly impaired immune system, since infection often results in a lethal infection, associated with chronic or disseminated virus replication (Ehtisham *et al*, 1993, Cardin *et al*, 1996, Weck *et al*, 1996 and Nash and Dutia personal communication). The lethality of these infections could be potentially circumvented with the application of an effective productive replication inhibitor, such as 4'-s-EtdU.

5.1.4 **The use of SCID mice as a model for EBV associated LPD**.

SCID mice have no adaptive immune system and do not reject allogeneic or xeno-tissue grafts. Humanised SCID mice have been used to model EBV associated LPD development. Injection of PBLs from healthy EBV sera positive individuals into SCID mice results in up to approximately 90% of recipient mice developing EBV positive B-cell LPD, after 1 to 3 months (Cannon *et al*, 1990 and Rowe *et al*, 1991). However, latently infected human B-cells alone, do not appear to efficiently induce LPD in immunodeficient mice. Injection of SCID mice with purified B-cells from EBV positive human donors, leads low efficiency LPD development. Furthermore, similar results were found after injection of PBLs from EBV positive human donors into immunodeficient mouse strains which accept B-cell but not T-cell xenografts, such as beige/nude/xid (BNX) mice. In these models of EBV associated LPD development, the further addition of T-cells, either CD4 or CD8, or exogenous virus was required for efficient LPD development (Dosch *et al*, 1991 and Veronese *et al*, 1992).

5.1.5 **The potential role of herpesvirus replication inhibitors in the study of MHV-68 associated lymphoproliferative disease**

Gammaherpesvirus associated LPD and neoplastic disorders have predominately been associated with immune disorders. This has been demonstrated by the high prevalence of both EBV associated LPD and BL and KSHV associated KS in immunosuppressed transplant patients and AIDS patients (see introduction). It is

therefore likely that MHV-68 associated lymphomas and LPD will also be associated with immune disorders. Many inbred and transgenic mice have been bred, that have components of the immune system made dysfunctional. Mice have been generated which have disrupted antigen presentation, such as MHC class II knock out mice, that lack specific killing mechanisms, such as fas and perforin knockout mice, that lack a specific cytokine, such as IL-6 and INFγ knock out mice or that lack a specific cytokine receptor such as INF-α/-β or INF-γ receptor knock mice. Other mice have been generated which lack cellular components of the immune system, such as beige mice, which lack natural killer cells, μMT mice which lack B-lymphocytes, nude mice which lack T-lymphocytes and severely combined immune deficient (SCID) mice which lack both B and T lymphocytes.

5.1.6 **Transient CsA induced immunosuppression of MHV-68 infected C57BL/6 mice.**

In an attempt to replicate the earlier work carried out by Dr Sunil-Chandra *et al*, 1994, 190 C57BL/6 female mice, 3 to 4 weeks of age were split into 2 groups. One consisted of 126 mice, which were infected with $4x10^5$ pfu MHV-68 (in 0.2 ml), via the intra peritoneal route. A second group consisted of 64 mice, which were injected (ip) with 0.2 ml BHK homogenate. At day 17 post infection, 4 mice were sampled from each group to assess the establishment of infection. At day 18 post infection, group 1 mice were divided into 2 groups (groups 1.A and 1.B) of 61 mice. The mice in group 1.2 and group 2 were injected (ip) with 0.2 ml CsA (100mg/kg mouse) and the mice in group 1.1 were injected (ip) with 0.2 ml olive oil. Further administration of CsA (50mg/kg) or olive oil (by ip injection) took place on days 21, 24 and 27 post infection. Four mice from each group were sampled on day 24 post infection and a further 3 mice from each group were sampled on day 37 post infection. The remaining mice remained under observation for signs of tumour development or general distress, for up to 2 years post infect. After 2 years the experiment was terminated and the mice killed.

5.1.7 **The FACS analysis carried on splenocytes from MHV-68 infected C57BL/6 mice immunosuppressed CsA.**

FACS analysis using, monoclonal antibodies specific to the CD4, CD8 and B220 antigens, was performed on the splenocyte preparations derived from all the mice sampled at days 17, 24 and 37 post infection (*see figure 5.1.1*). The infected mice underwent splenomegaly. This resulted in a disproportionate increase in the proportion of T-cells present in the spleen, especially CD8 T-cells, which was not affected by the CsA treatment. The mean percentage of CD8 positive splenocytes was significantly higher in the infected (group 1) mice than in the uninfected (group

Figure 5.1.1

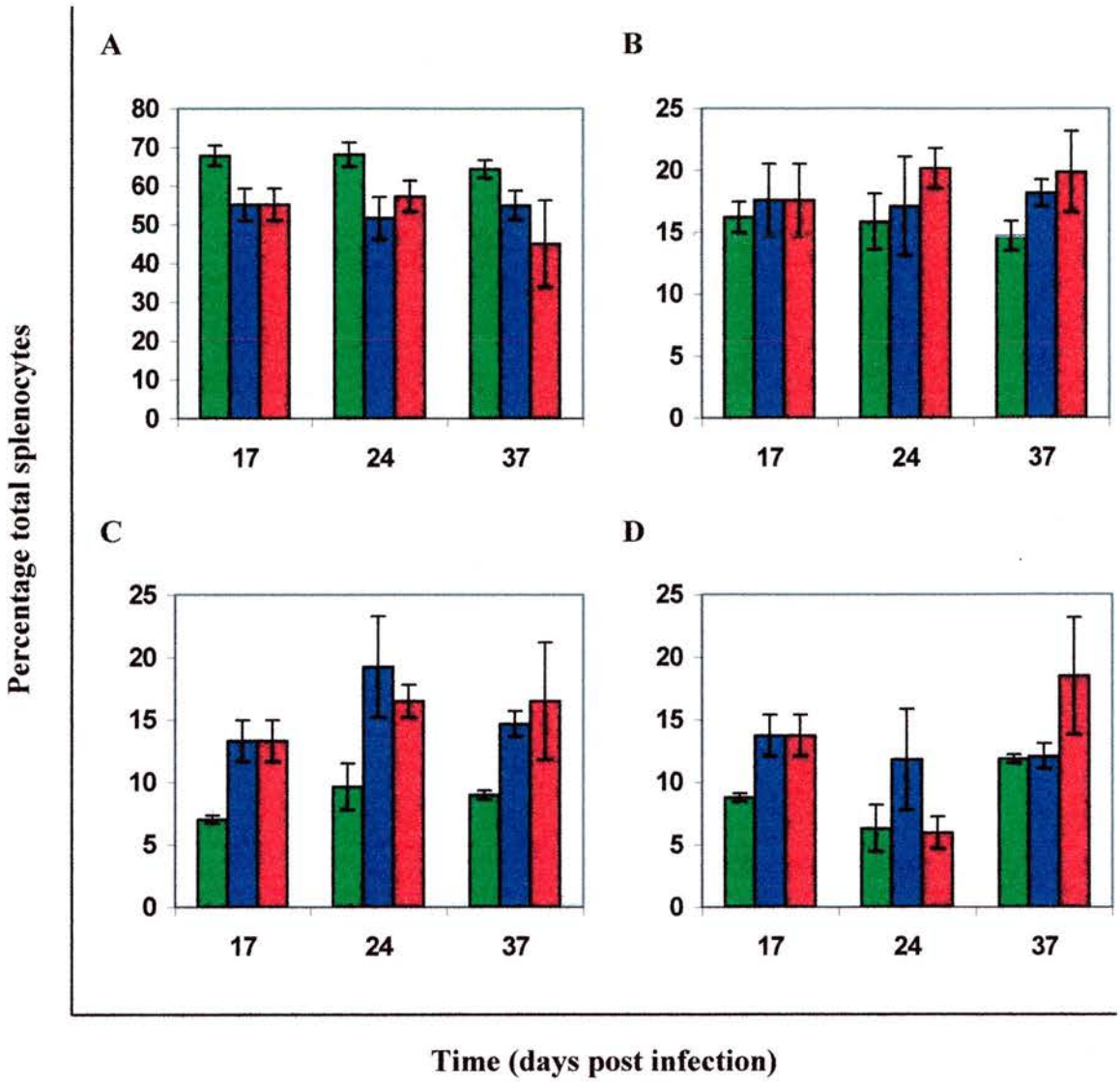


Figure 5.1.1 The relative proportions of different lymphocyte sub-populations present in the spleens of C57BL/6 mice infected with MHV-68 (ip) and then either immunosuppressed with CsA from day 17 post infection (red) or left untreated (blue). These were compared to non-infected mice which also immunosuppressed with CsA from day 17 post infection (green). The proportion of the different lymphocyte sub-populations was determined by FACS analysis. Monoclonal antibodies specific for B220 (A), CD4 (B), CD8 (C) were used to determine the proportion of B-cells, CD4 T-cells and CD8 T-cells respectively. The proportion of null cells (D) was determined by the proportion of splenocytes cumulatively negative for CD4, CD8 and B220 cell markers. The data values are represented by arithmetic means and the error bars represent the standard deviations. The level of detection was approximately 1%.

2) mice, at all 3 time points tested (P=0.005, 0.013 and 0.0019 respectively, by student T-test). The mean percentage of CD8 positive splenocytes was also significantly higher in the infected CsA treated (group 1.B) mice than in the uninfected (group 2) mice (P=0.0018 and 0.049 respectively, by student T-test), at both days 24 and 37 post infection (*see figure 5.1.1B*). The mean percentages CD4 positive splenocytes was also higher in both the infected (group 1.A) and the infected CsA treated (group 1.B) mice, at all 3 time points, than in the uninfected (group 2) mice (*see figure 5.1.1A*). However, the differences were not statistically significant.

Conversely, the proportion of B-cells present in the spleens of the infected mice was suppressed during splenomegaly. This again was not affected by the CsA treatment. The mean percentage of B220 positive splenocytes was significantly lower in the non CsA treated infected (group 1.A) mice than in the uninfected (group 2) mice, at all 3 time points tested (P=0.0035, 0.0065 and 0.015 respectively, by student T-test). The mean percentage of B220 positive splenocytes was also significantly lower in the infected CsA treated (group 1.B) mice, at both days 24 and 37 post infection, than in the uninfected (group 2) mice (P=0.0018 and 0.049 respectively, by student T-test) (*see figure 5.1.1C*). There were no significant differences between the percentages of CD4, CD8 and B220 positive splenocytes observed in the infected (group 1.A) mice and the infected CsA treated (group 1.B) mice at either of the 2 time points tested.

5.1.8 **The effect of CsA immunosuppression on the total numbers splenic lymphocyte sub-populations in MHV-68 infected C57BL/6 mice.**

The number of splenocytes were evaluated for each mouse sampled on days 17, 24 and 37 post infection (*see figure 5.1.2A*). On day 17 post infection, the number of splenocytes observed in the infected mice was almost twice that of the uninfected mice ($1.5x10^8$ leukocytes as opposed to $0.8x10^8$ leukocytes, P=0.0032 by student T-test). The number of splenocytes remained higher in infected compared to the uninfected mice at day 24 ($1.15x10^8$ leukocytes as opposed to $0.9x10^8$ leukocytes).

Figure 5.1.2

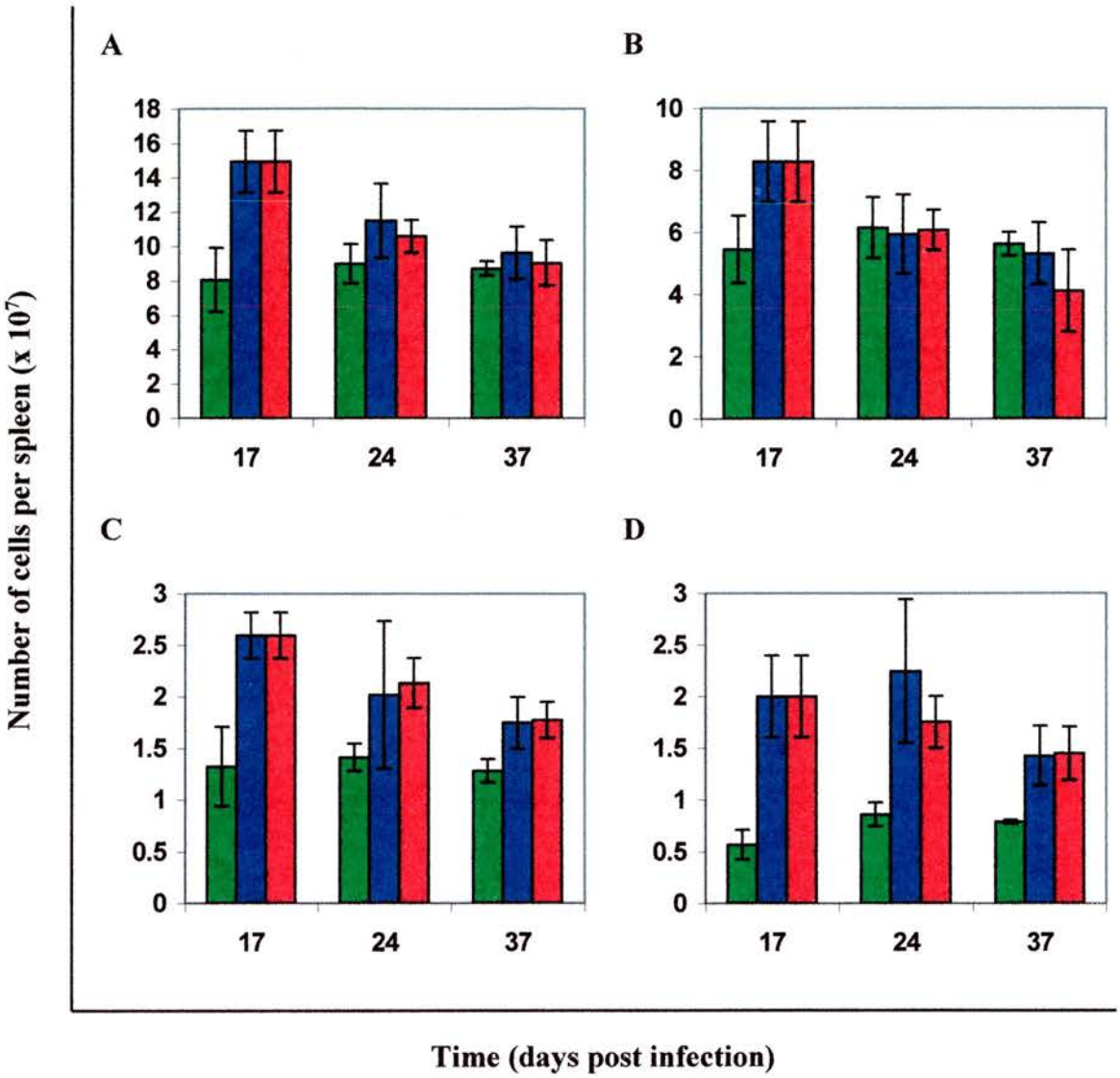


Figure 5.1.2 The total number of splenocytes and lymphocyte sub-populations present in the spleens of C57BL/6 mice infected with MHV-68 (ip). The infected mice were either immunosuppressed with CsA, from day 17 post infection, (red) or left untreated (blue). The infected mice were compared to non-infected mice which were also immunosuppressed with CsA, from day 17 post infection, (green). The total number of splonocytes present per spleen (A) was determined by haemocytometer counts of water lysed splenocyte preparations. The proportion of the different lymphocyte sub-populations was determined by FACS analysis. Monoclonal antibodies to B220 (B), CD4 (C), CD8 (D) were used to determine the proportion of B-cells, CD4 T-cells and CD8 T-cells respectively. The individual numbers of B-cells, CD4 T-cells and CD8 T-cells was calculated from the total number of splenocytes for each mouse. The data values are represented by arithmetic means and the error bars represent the standard deviations. The level of detection was 1% of the total splenocyte count.

However, the difference was no longer statistically significant, remained so for all later time points.

The total numbers of CD4 T-cells, CD8 T-cells and B220 positive B-cells was determined (per spleen) for each of the mice sampled on days 17, 24 and 37 post infection (*see figure 5.1.2B, C and D*). The total number of T-cells observed in the infected mice remained significantly higher than in the uninfected mice, even after the splenomegaly had subsided. There were significantly higher numbers of CD8 T-cells observed in both the infected (group 1A) mice (P= 0.0065, 0.029 and 0.022 respectively, by student T-test), and the infected CsA treated (group 1B) mice (P= 0.0029 and 0.015 respectively, by student T-test), than in the uninfected (group 2) mice, on all 3 time points tested, days 17, 24 and 37 post infection. There were also significantly higher numbers of CD4 T-cells observed in both the infected (group 1A) mice (P= 0.0045, 0.19 and 0.03 respectively, by student T-test) and the infected untreated (group 1B) mice (P= 0.0064 and 0.011 respectively, by student T-test) than in the uninfected (group 2) mice.

However, the total number of B-cells observed in the infected (group 1A) mice was only significantly higher than the uninfected (group 2) mice, at the first time point, day 17 post infection, during peak splenomegaly (P=0.02 by student T-test). There was no significant differences between the number of B-cells observed in either the infected (group 1A) or infected CsA treated (group 1B) mice, than the uninfected (group 2) mice, at either of the latter 2 time points, days 24 and 37 post infection.

Once again, the CsA treatment appeared to have no significant effect on the numbers of splenic B and T lymphocytes, in the infected mice. There was no significant difference in the numbers of CD4, CD8 or B220 positive splenocytes cells observed in the infected (group1A) mice and the infected CsA treated (group 1B) mice at either of the 2 time points tested, days 24 and 37 post infection.

5.1.9 **The effect of CsA immunosuppression on latent and productive virus replication in MHV-68 infected C57BL/6 mice.**

The number of latently infected splenocytes was determined, by infectious centre assay, for the infected (group 1A), infected CsA treated (group 1B) and uninfected (group 2) mice (*see figure 5.1.3*). Infectious centres were detected only in the infected mice. At day 24 days post infection (day 6 post CsA treatment), the infectious centre titre observed in the CsA treated (group 1B) mice was significantly higher than both the untreated (group 1A) mice (P=0.01 by student T-test). The infectious centre titre observed in the CsA treated (group 1B) mice was also significantly higher than observed in the mice during peak splenomegaly, sampled prior to treatment, on day 17 post infection (P=0.49 by student T-test). However, by day 37 post infection (10 days after the last CsA injection) both the treated and untreated mice (groups 1A and 1B) had decreased to an equally low titre. Only 2 of the 3 mice tested from both groups gave rise to infectious centres with the mean titres showing no significant difference (P=0.87 by student T-test).

To determine if an increase in productive viral replication had occurred in the CsA treated mice, infectious virus assays were carried out on the lungs, spleens and adrenal glands of the infected mice (groups 1A and 1B), sampled at days 17, 24 and 37 post infection. No infectious virus was detectable in any of the 3 organs tested in any of the mice, by direct plaque assay (*data not shown*).

5.2.1 **The effect of CsA immunosuppression on LPD development in MHV-68 infected C57BL/6 mice.**

Through out the course of the next 2 years the mice were regularly checked for signs of illness or tumour development. Although a small percentage showed signs of illness, none of these animals showed signs of tumour development. Infectious virus and infectious centre assays were carried out on the lungs and spleens respectively (*data not shown*). None of the mice that were killed due to signs of illness contained

Figure 5.1.3

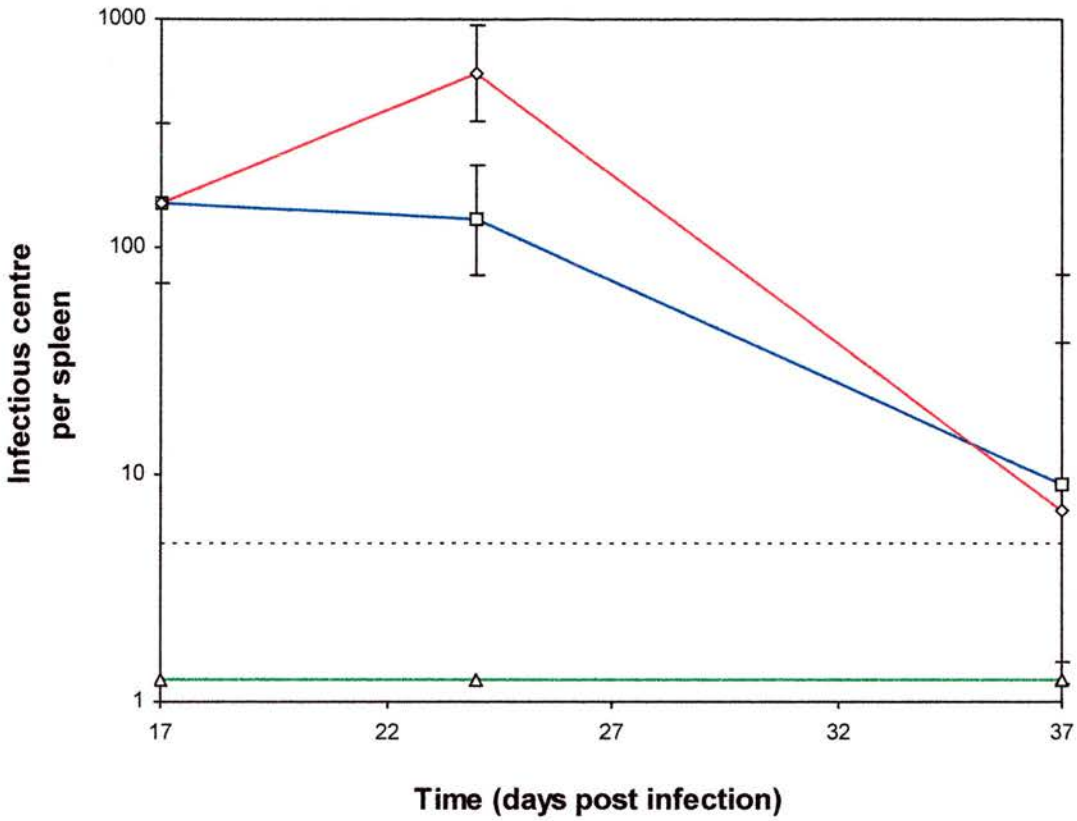


Figure 5.1.3 The levels of viral latency in MHV-68 infected and uninfected mice, as determined by infectious centre assay. The mice were infected with $4x10^5$ pfu MHV-68, via intra-peritoneal injection. The infected mice were either CsA treated (red) or left untreated (Blue). All the uninfected mice (green) were also CsA treated. CsA treatment (50mg/Kg) was initiated on day 18 post infection and was carried out, by intra-peritoneal injection, at 3 day intervals. The last CsA injection took place at day 27 post. Four mice were sampled at days 17 and 24 post infection and 3 mice were sampled at day 37 post infection. The data is represented by geometric means and the error bars represent the standard deviations.

detectable levels of infectious virus in the lungs or had elevated splenic infectious centre titres. After 2 years the mice were killed and spleens and mesenteric lymph nodes removed. None resembled either the high or low grade lymphomas previously described by Sunil-Chandra *et al*, 1994.

5.2.2 **The adoptive transfer of syngeneic splenocytes into SCID mice from MHV-68 infected donors.**

A separate set of experiments were carried out to determine whether MHV-68 infected B-cells could give rise to lymphomas or lymphoproliferative disease in SCID mice. Fourteen female BALB/c mice, 3 to 4 weeks of age, were infected (ip) with $4x10^5$ pfu MHV-68 in a pilot study. The mice were put on drinking water supplemented with 4'-s-EtdU on day 6 post infection and killed on day 11 post infection. The ficoll purified splenocytes were injected (ip) into 2 groups of SCID mice ($7x10^7$ cells per mouse), 4 to 5 weeks of age. Groups 1 mice were placed on drinking water supplemented with 4'-s-EtdU (0.3 mg/ml) from 2 days prior to adoptive transfer and remained until the experiment was terminated, while the group 2 mice remained untreated. The 2 Groups were further split into a CD8 depleted (1B and 2B) and undepleted (1A and 2A) group. Groups 1B and 2B received splenocytes mixed with anti-CD8 antibody (1mg / $7x10^7$ splenocytes) and on day 3 post adoptive transfer, were injected (ip) with a further dose of CD8 depleting antibody (1mg / mouse). On day 21 days post adoptive transfer the mice were killed for analysis.

5.2.3 **FACS analysis carried out on splenocytes from SCID mice following the adoptive transfer of cells from MHV-68 infected donors.**

FACS analysis was carried out on donor splenocytes and splenocyte preparations derived from the SCID mice sampled on day 21 post adoptive transfer (*see figure 5.2.1*). The proportions of CD4, CD8 and IgM positive splenocytes were lower in the SCID mice, than the donor splenocytes used in the adoptive transfer. On average, there was a 10 fold decrease in the proportion of IgM positive splenocytes

Figure 5.2.1

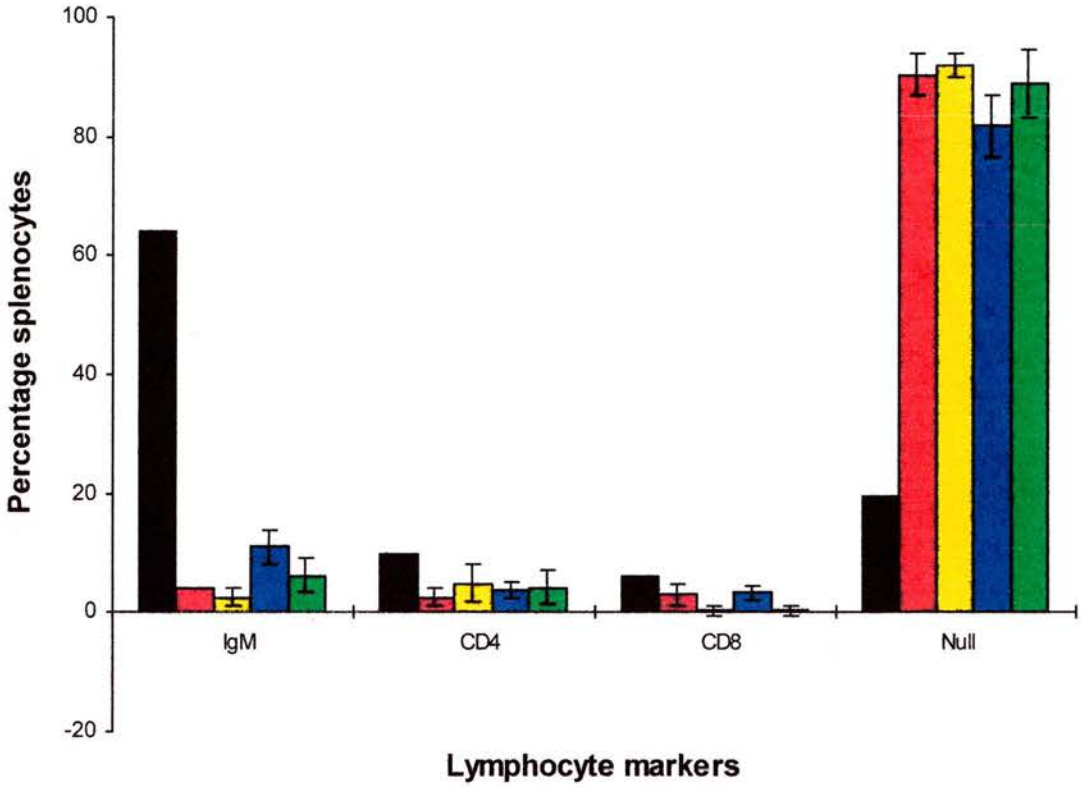


Figure 5.2.1 The relative proportions of different splenic lymphocytes sub-populations observed in SCID mice adoptive transfer recipients. The SCID mice all received $2x10^7$ pooled splenocytes (black) from MHV-68 infected (ip) BALB/c mice, sampled day 11 post infection. Group 1 mice were placed on drinking water supplemented with 4'-s-EtdU (0.3 mg/ml), 2 days prior to adoptive transfer, while the group 2 mice remained untreated. Half the mice from both groups received CD8 T-cell depleted splenocytes (B) whilst the other half received non-depleted splenocytes. Each of the resultant 4 groups of mice, 1A, 1B, 2A and 2B (red, yellow, blue and green respectively) contained 3 mice which were all sampled at day 21 post adoptive transfer. The proportions of CD4, CD8 and IgM positive lymphocytes, present per mouse spleen, was determined by FACS analysis and the proportion of null cells was derived from the cumulative proportion of CD4, CD8 and IgM negative splenocytes. The data is represented by arithmetic means and the error bars represent the standard deviations. The limit of detection was 1%.

in the SCID mice, as compared to the donor splenocytes (6% as opposed to 64% respectively). The decrease in CD4 and CD8 positive splenocytes was an average 2.5 and 2 fold respectively, in the SCID mice as compared to the donor splenocytes. As a result, the largest sub-population of splenocytes observed in the SCID mice recipients were null cells (cells negative for CD4, CD8 and IgM), which were approximately 90% of the total splenocytes. This compares to a null cell population of only 20% of the total donor cells. The levels of CD8 positive splenocytes present in the CD8 depleted groups of SCID mice (1B and 2B) were both indistinguishable from the negative controls, and below accurate evaluation by FACS analysis.

The total number of splenocytes and the number of CD4, CD8 and IgM positive cells was determined for each spleen (*see figure 5.2.2*). The number of splenocyte per spleen in the CD8 depleted SCID mice recipients (groups 1B and 2B) were both lower than the undepleted groups (1A and 1B). However, the differences were not significant, and the average splenocyte counts for all the SCID mouse groups, totalled approximately 1/10 of the number, injected during adoptive transfer. Despite the numbers of cells being relatively low, significant numbers of CD4 and IgM positive splenocytes were detectable in all the recipient SCID mice (averaging, $2.4x10^5$ and $4x10^5$ cells per spleen, respectively), and $2.1x10^5$ CD8 positive cells were observed in the non-depleted SCID recipients (groups 1A and 2A).

5.2.4 **The levels of latent and productive virus replication in SCID mice receiving splenocytes from MHV-68 infected donors.**

Latently infected cells could be detected in all the SCID mice except 1 mouse in group 1A, by infectious centre assay. The total number of infectious centres per spleen was relatively low (at least 10 fold lower than the number injected in during adoptive transfer) in all the SCID mice groups (*see figure 5.2.3A*). However, the number of infectious centres relative to the number of total splenocytes or B-cells (IgM positive splenocytes) was directly comparable to the levels seen in the donor

Figure 5.2.2

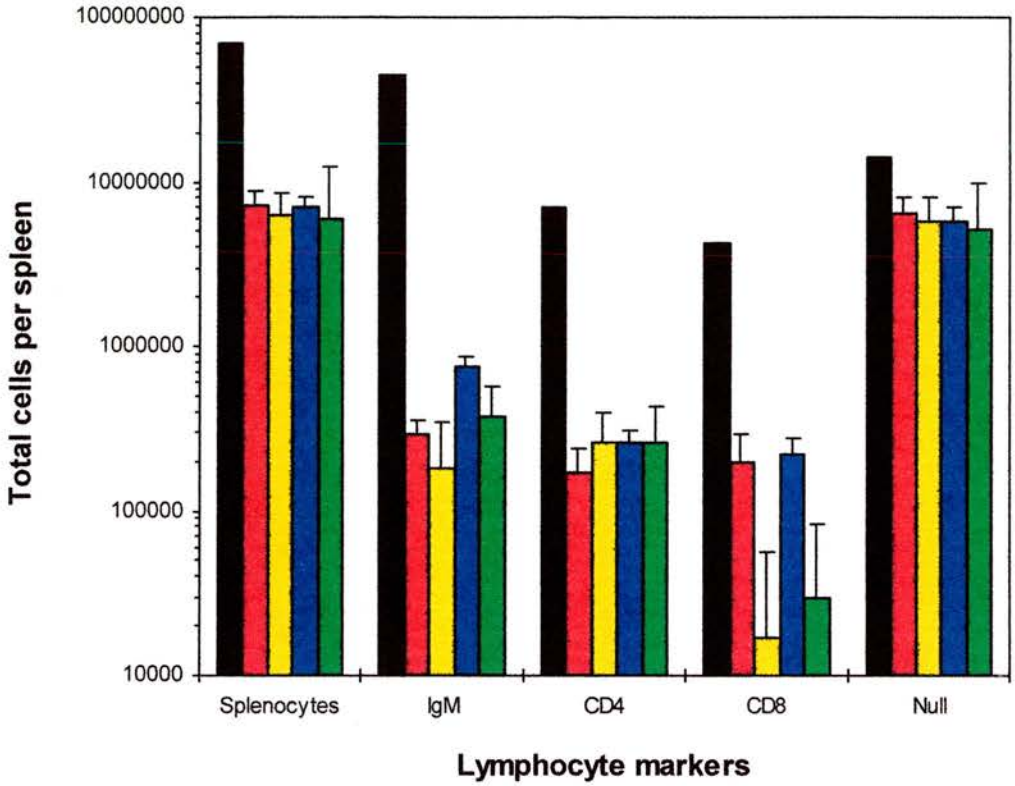
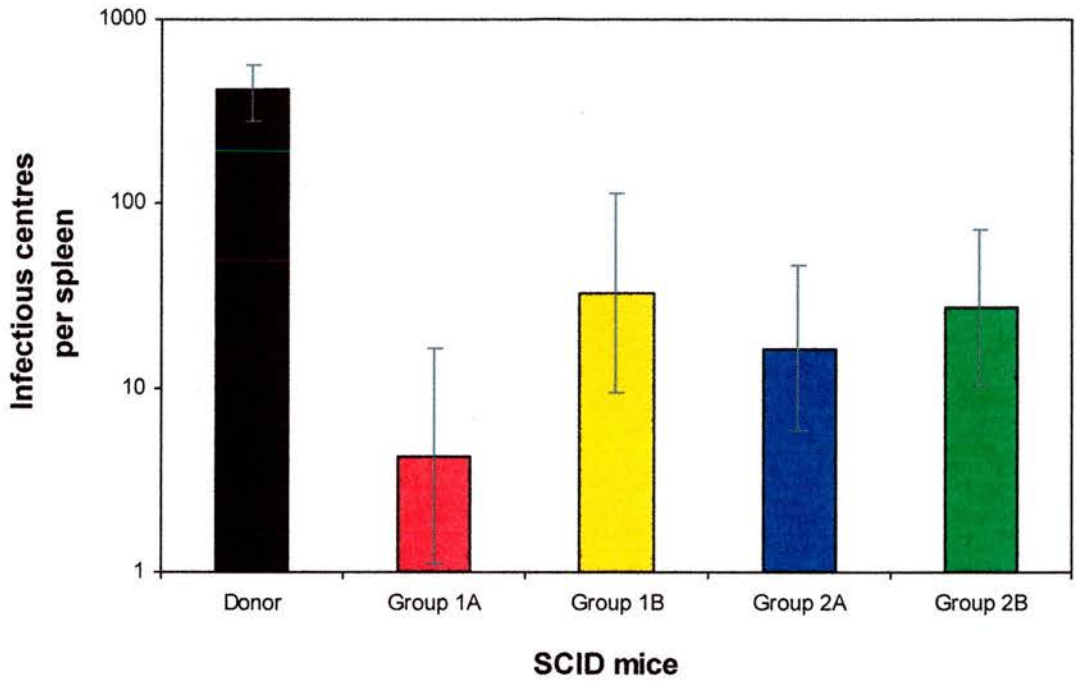


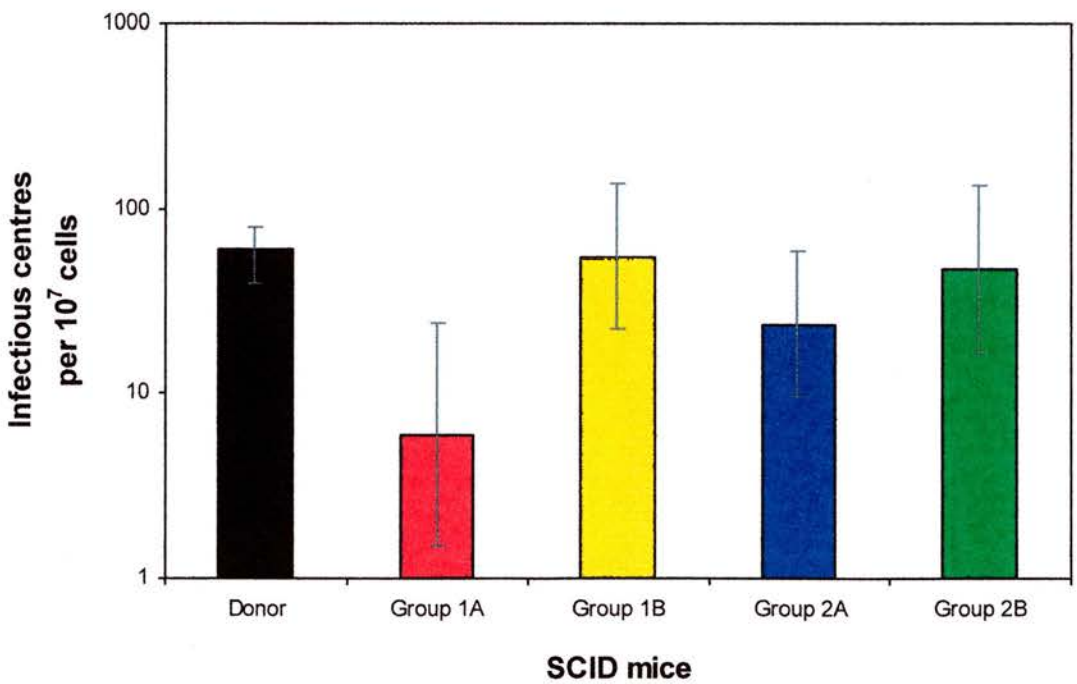
Figure 5.2.2 The total numbers of splenocytes and CD4, CD8 and IgM positive lymphocytes present in the spleens of SCID mouse adoptive transfer recipients. The SCID mice all received $2x10^7$ pooled splenocytes (black) from MHV-68 infected (ip) BALB/c mice, sampled day 11 post infection. Group 1 mice were placed on drinking water supplemented with 4'-s-EtdU (0.3 mg/ml), 2 days prior to adoptive transfer, while the group 2 mice remained untreated. Half the mice from both groups received CD8 T-cell depleted splenocytes (B) whilst the other half received non-depleted splenocytes. Each of the resultant 4 groups of mice, 1A, 1B, 2A and 2B (red, yellow, blue and green respectively) contained 3 mice which were all sampled at day 21 post adoptive transfer. The number of splenocytes per mouse was evaluated using a haemocytometer and was multiplied by the proportion of CD4, CD8 and IgM positive lymphocytes, as determined by FACS analysis. The data is represented by arithmetic means and the error bars represent the standard deviations. The limit of detection for the SCID mice was approximately $8x10^4$ cells.

Figure 5.2.3

A



B



C

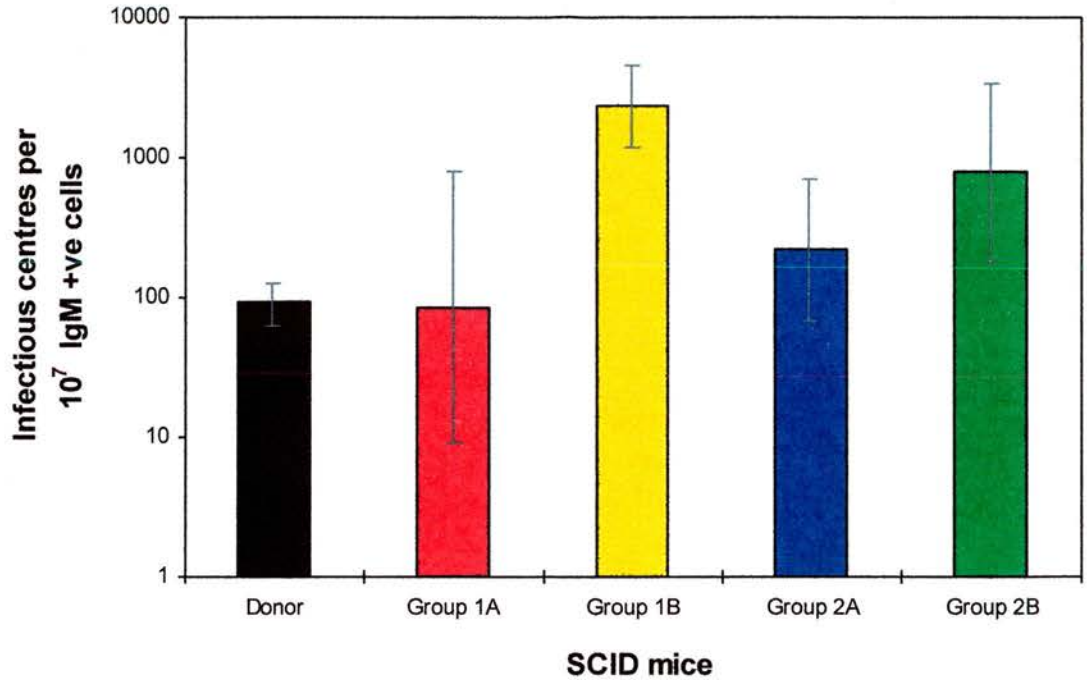


Figure 5.2.3 The latent virus titre, per spleen (A), relative to the number of splenocytes present per spleen (B) and relative to the number of splenic B-lymphocytes present per spleen (C), in SCID mice recipients of adoptive transfers. The SCID mice all received $2x10^7$ pooled splenocytes (black) from MHV-68 infected (ip) BALB/c mice, sampled day 11 post infection. Group 1 mice were placed on drinking water supplemented with 4'-s-EtdU (0.3 mg/ml), 2 days prior to adoptive transfer, while the group 2 mice remained untreated. Half the mice from both groups received CD8 T-cell depleted splenocytes (B) whilst the other half received non-depleted splenocytes. Each of the resultant 4 groups of mice, 1A, 1B, 2A and 2B (red, yellow, blue and green respectively) contained 3 mice which were all sampled at day 21 post adoptive transfer. Levels of splenic latency were evaluated by infectious centre assay. The data is represented by geometric means and the error bars represent the standard deviations.

splenocytes (*see figure 5.2.3B and C*). There was no significant differences in infectious centre titre between the 4'-s-EtdU treated mice and the non-treated mice. There was also no significant differences between the CD8 depleted and no depleted groups, despite both CD8 depleted groups having a greater average infectious centre titre than the respective undepleted groups.

Infectious virus assays were carried out on the donor splenocytes, and the lungs, spleen and adrenal glands from the sampled SCID mice. No infectious virus could be detected in any of the mice organs tested nor in the donor splenocytes (*data not shown*).

5.2.5 **The MHV-68 infection of reconstituted SCID mice.**

Studies into the transforming activities of EBV, have shown that inoculation of cell free infectious virus into humanised SCID mice that lack T-cells, greatly increases the incidence of LPD development. The implication from this work is that latently infected B-cells contained within PBLs taken from healthy EBV sera positive individuals, do not directly give rise to LPD. Although the SCID mice in the previous experiment received splenocyte adoptive transfers from MHV-68 infected mice that contained both CD4 and CD8 T-cells, no free virus was detected either in the splenocytes constituting the adoptive transfer or in the SCID mice after reconstitution. Therefore to determine whether LPD could be induced in reconstituted SCID mice following infection with cell free virus, groups of SCID mice were adoptively transferred with CD8 depleted and non-depleted splenocytes from both infected and uninfected BALB/c donor and then infected (ip) with cell free virus.

Twenty one female BALB/c mice, 3 to 4 weeks of age were infected (ip) with $4x10^5$ pfu MHV-68 while the splenocytes from a further 30 mice were injected (ip) into 2 groups of 16 SCID mice ($4x10^7$ splenocytes per mouse, in 0.4ml). The group 1 SCID mice received whole splenocyte adoptive transfers. The group 2 mice received

whole splenocyte adoptive transfers but were then CD8 depleted via injected (ip) with anti-CD8 monoclonal antibodies (1mg/4x10^7 cells). Twenty four hours post adoptive transfer, the non-CD8 depleted (group 1) mice were divided into a further 2 groups (1A and 1B) of 8 mice. The group 1B mice along with the CD8 depleted (groups 2) mice were infected (ip) with 4x10^4 pfu MHV-68. On day 3 post adoptive transfer, the group 2 mice were injected with a further dose of anti-CD8 monoclonal antibody (1 mg/mouse). On day 6, the CD8 depleted (group 2) mice were split into a further 2 groups (2A and 2B) of 8 mice. The group 2B mice were put on drinking water supplemented with 4'-s-EtdU (0.3mg/ml), for the remainder of the experiment. The group 2A and group 1 mice remained on non-supplemented drinking water (*see figure 5.3.1 and table 5.1 for a summary of the different SCID mouse groups*).

On day 10, the MHV-68 infected BALB/c mice were killed and the splenocytes from which, injected (ip) into 2 groups (3 and 4) of 8 and 16 SCID mice respectively (4x10^7 splenocytes per mouse, in 0.4ml) and a group of 8 uninfected BALB/c mice controls (group 6). The group 4 mice were CD8 depleted via injection (ip) with anti-CD8 monoclonal antibodies (1mg/4x10^7 cells), whilst the group 3 mice remained undepleted. This left a group of 8 SCID mice (group 5), that did not receive an adoptive transfer. Half the group 4 mice (group 4B) along with all the non-reconstituted (group 5) SCID mice were then injected (ip) with 4x10^4 pfu MHV-68. The remaining group 4 mice (group 4A) SCID mice were not super infected with MHV-68. On day 13, the CD8 depleted SCID mice (groups 4A and 4B) were injected (ip) with a second dose of anti-CD8 antibody (1 mg/mouse). On day 16, the MHV-68 infected non-reconstituted (group 5) SCID mice were split into a further 2 groups of 4 mice (groups 5A and 5B). The group 5B mice, along with the superinfected CD8 depleted (group 4B) SCID mice were put on drinking water supplemented with 4'-s-EtdU (0.3mg/ml), which they remained on through out the experimental time course. The group 5A mice remained on normal drinking water. Mice were sampled at days 7, 36 and 60 post adoptive transfer (*see figure 5.3.1 and table 5.1 for a summary of the different SCID mouse groups*).

Figure 5.3.1

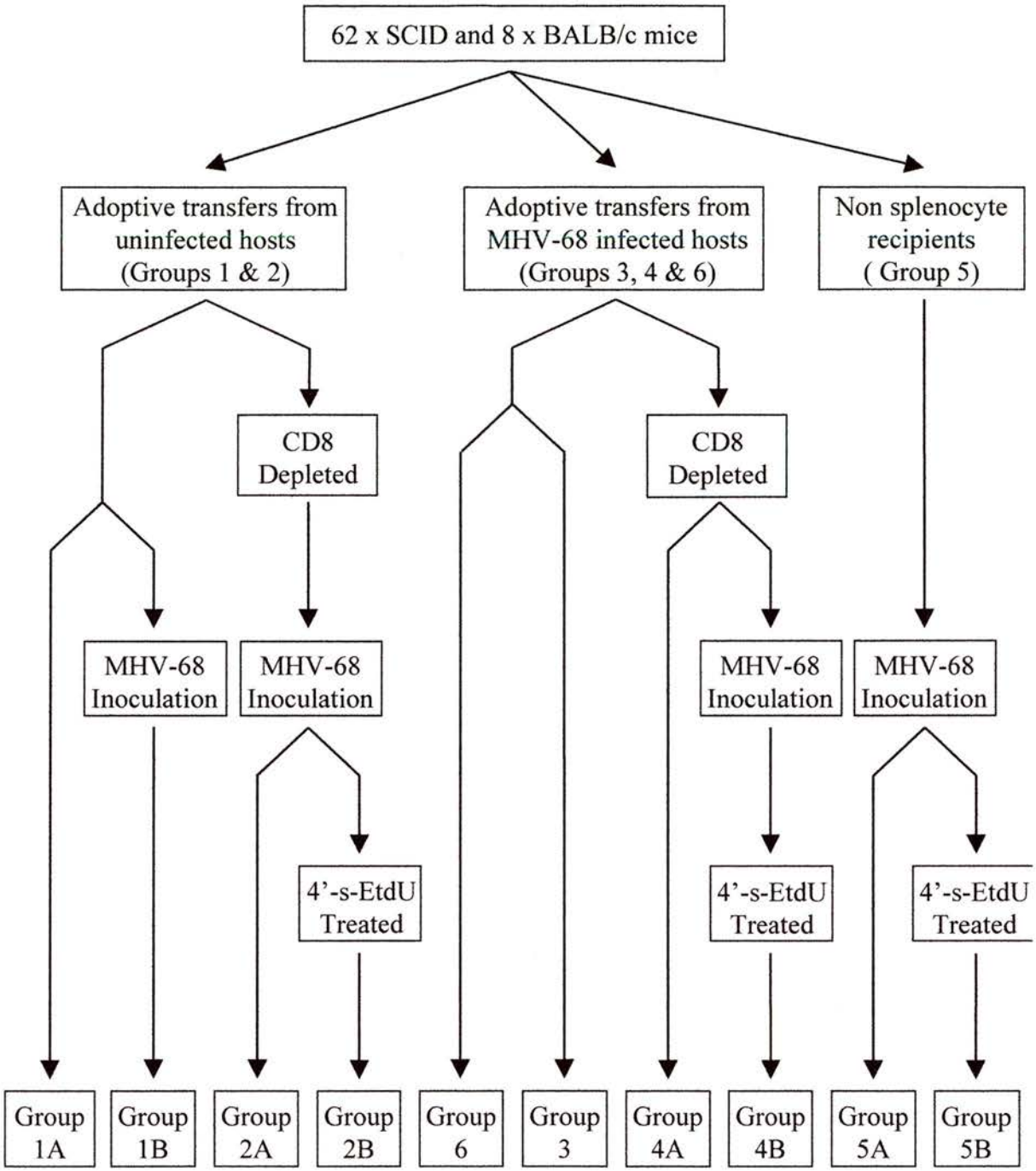


Figure 5.3.1 The summarised procedures carried out on the different groups of recipient mice in sections 5.2.5 and 5.2.7 (Also see Table 5.1). All the groups of mice received an adoptive transfer of splenocytes (except for the group 5 mice), via intra-peritoneal injection. The donor mice were all sex matched, syngeneic BALB/c mice. All the groups comprised of 8 x SCID mice except for the group 6 mice (which comprised of 8 x BALB/c mice) and groups 5A and 5B (which comprised of 8 x SCID mice). CD8 depletion was carried out via intra-peritoneal injection of anti-CD8 monoclonal antibodies and days 0 and 3 post adoptive transfer. MHV-68 inoculation was carried out on day 1 post adoptive transfer, via intra-peritoneal injection of $4x10^5$ pfu MHV-68. 4'-s-EtdU treatment initiated on day 6 post adoptive transfer and delivery occurred via drinking water.

Table 5.1 **The summarised procedures carried out on the different groups of splenocyte adoptive transfer recipient mice in sections 5.2.5 and 5.2.7**

Group		Mice	Donor BALB/c Mice	CD8 Depletion	Exogenous Infection	4'-s-EtdU Treated
1	A	8xSCID	Uninfected	-	-	-
	B	8xSCID	Uninfected	-	+	-
2	A	8xSCID	Uninfected	+	+	-
	B	8xSCID	Uninfected	+	+	+
3		8xSCID	*Infected	-	-	-
4	A	8xSCID	*Infected	+	-	-
	B	8xSCID	*Infected	+	+	+
5	A	4xSCID	-	-	+	-
	B	4xSCID	-	-	+	+
6		8xBALB/c	*Infected	-	-	-

Key: - ~ procedure not done, + ~ procedure carried out. * Donor BALB/c mice were infected with $4x10^5$ pfu MHV-68 (ip) 11 days prior to the mice being killed and the splenocyte adoptive transfers taking place.

5.2.6 **Retention of lymphocytes in the SCID mouse recipients of splenocytes from uninfected BALB/c mice.**

The proportion of CD4 T-cells, CD8 T-cells and IgM positive B-cells present in the uninfected donor splenocytes and their respective SCID mouse recipients (groups 1 and 2) was evaluated by FACS analysis, at days 37 and 60 post adoptive transfer. A significant proportion of the splenocytes derived from all the SCID mice recipients were CD4 positive (*see figure 5.3.2A*). The proportion of CD8 splenocytes in the non-depleted mice (groups 1A and 1B) was equal or greater than the proportion seen in the donor splenocytes (*see figure 5.3.2B*). However, in the CD8 depleted SCID mice (groups 2A and 2B) the number of CD8 cells was below the level of detection by FACS analysis. The numbers of IgM positive B-cells, in all the groups of reconstituted SCID mice was low, relative to the donor splenocytes (*see figure 5.3.2C*). This was especially true for the CD8 depleted mice (groups 2A and 2B). The combined average proportion of IgM positive cells (for all 4 groups) was under 5%, at both day 36 and 60 post adoptive transfer. As was the case in the pilot study, the single largest sub-population of splenocytes were the null (CD4, CD8 and IgM negative cells, calculated assuming the cells positive for CD4, CD8 and IgM were mutually exclusive) cells (*see figure 5.3.2D*). The proportion of null cells was higher in the CD8 depleted groups ranged (across the time point) from an average of 92% (+/- 6.6) in the group 2B mice to an average of 50% (+/- 20) in the group 1B mice.

5.2.7 **Virus latency in SCID mice reconstituted with uninfected splenocytes and infected post adoptive transfer.**

The level of viral latency in the spleen was assessed in the mice sampled at each time point with the infectious centre assay (*see figure 5.3.3*). At day 7 post adoptive transfer, relatively high titres of viral latency were detected in all 3 groups of infected mice (groups 1B, 2A and 2B). However, at day 36 and 60 post adoptive transfer, latent virus was barely detected (*see figure 5.3.3*). From the 6 mice sampled, at day 36, only 1 mouse (in group 2A) from the 3 infected SCID mouse

Figure 5.3.2

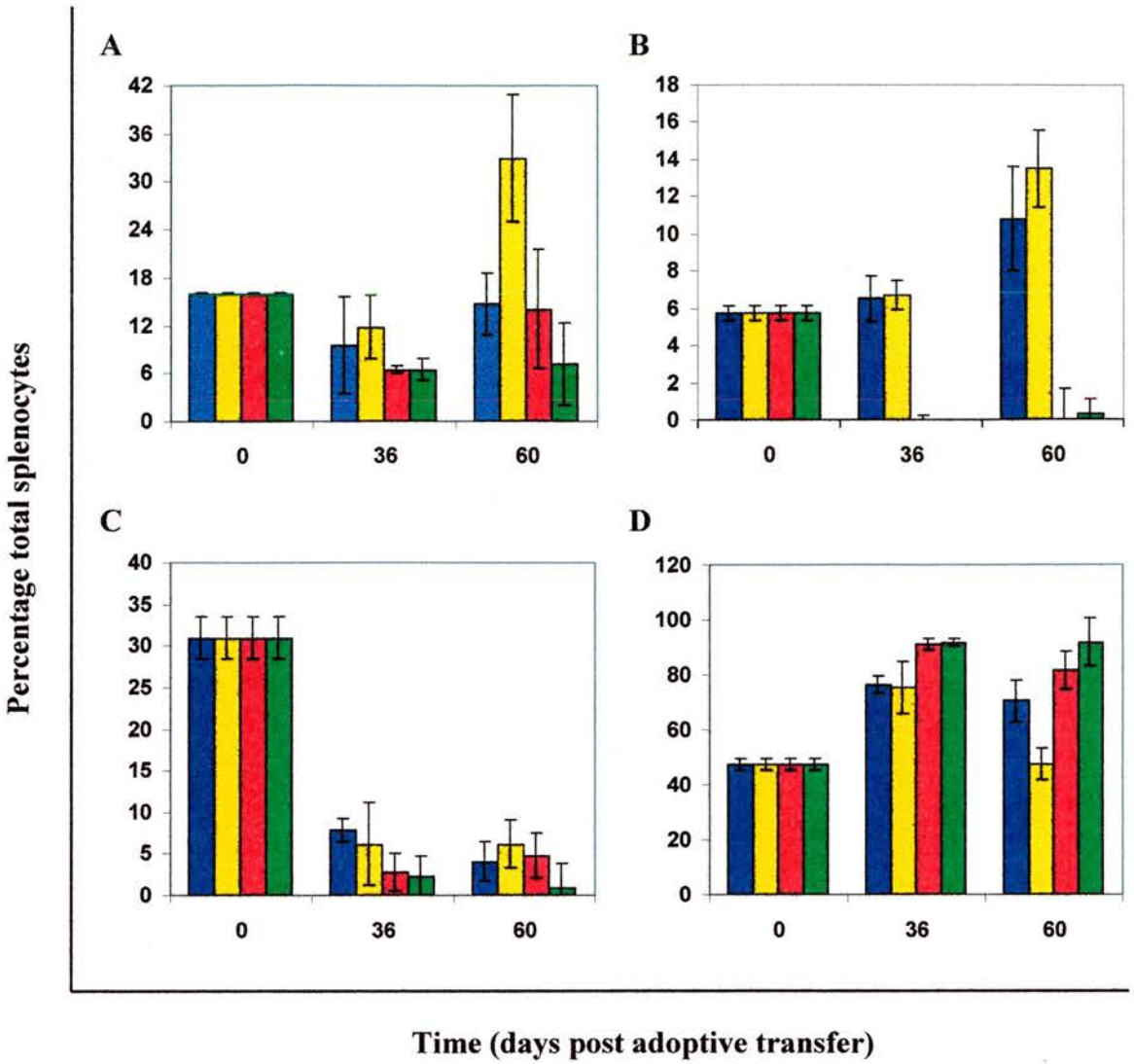


Figure 5.3.2 The relative proportion of CD4 (A), CD8 (B) IgM (C) and null positive splenocytes observed groups 1A, 1B, 2A, and 2B (blue, yellow, red and green respectively) splenocyte adoptive transfer recipient SCID mice. The mice in each group received $4x10^7$ splenocytes (pooled from uninfected BALB/c mice) via intra-peritoneal injection (0.4ml per mouse). Groups 2A and 2B received CD8 T-cell depleted splenocytes and groups 1B, 2A and 2B were infected with $4x10^4$ pfu MHV-68, via intra-peritoneal injection, 24 hours after the adoptive transfers. The group 2B mice were placed on drinking water supplemented with 4'-s-EtdU (0.3mg/ml) from day 6 post adoptive transfer. The proportions of CD4, CD8 and IgM positive splenocytes were determined by FACS analysis and the number of null cells was calculated as the cumulative proportion of CD4, CD8 and IgM negative cells. FACS analysis was carried out on the splenocyte preparations from the mice sampled on days 36 and 60 post adoptive transfer (2 and 4 mice respectively per group). The day 0 post adoptive transfer value represents the adoptive transfer splenocytes. The data is represented by arithmetic means and the error bars represent the standard deviations. The limit of detection was 1%.

Figure 5.3.3

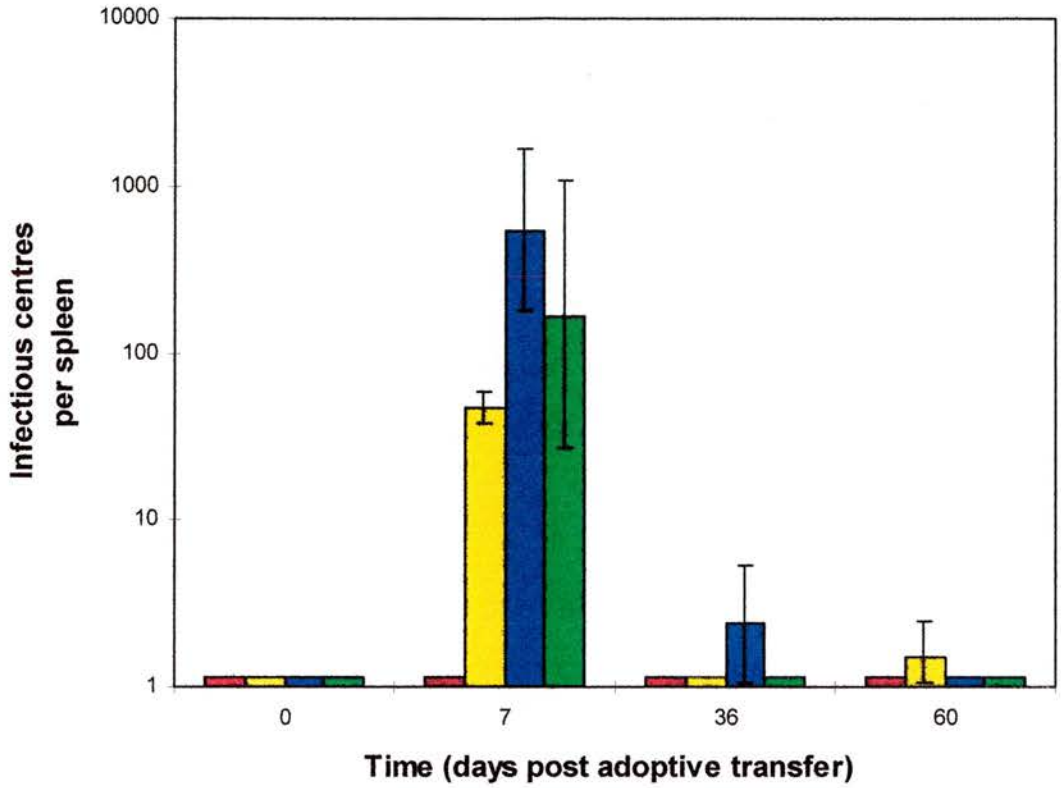


Figure 5.3.3 The levels of viral latency, as determined by infectious centre assay, in groups 1, 2, 3 and 4 (blue, yellow, red and green respectively) of the splenocyte adoptive transfer recipient SCID mice. The mice in each group received $4x10^7$ splenocytes (pooled from uninfected BALB/c mice) via intra-peritoneal injection (0.4ml per mouse). Groups 3 and 4 received CD8 T-cell depleted splenocytes. Groups 2, 3 and 4 were infected with $4x10^4$ pfu MHV-68, via intra-peritoneal injection, 24 hours after the adoptive transfers. The group 4 mice were placed on drinking water supplemented with 4'-s-EtdU (0.3mg/ml) from day 6 post adoptive transfer. Mice sampled on days 7, 36 and 60 post adoptive transfer (2, 2 and 4 mice respectively). The day 0 post adoptive transfer value represents the number of latently infected cells injected (ip) into each mouse. The data is represented by geometric means and the error bars represent the standard deviations.

groups (1B, 2A and 2B) had a detectable infectious centre titre. Further more from the 12 mice sampled at day 60, only 1 mouse (in group 1B) from the 3 infected groups had a detectable infectious centre titre. There was no significant increase in spleen cell numbers nor lymphoma development, in any of the mice over the time course (data not shown).

5.2.8 **Retention of lymphocytes in the SCID mouse recipients of splenocytes from MHV-68 infected BALB/c mice.**

The proportion of CD4 T-cells, CD8 T-cells and IgM positive B-cells was also evaluated by FACS analysis for the splenocytes derived from MHV-68 infected donors and their respective recipients (groups 3, 4A, 4B and the normal BALB/c recipients) sampled at days 37 and 60 post adoptive transfer. A significant proportion of CD4 T-cells were detected in the splenocytes derived from all the mice tested at every time point (*see figure 5.3.4A*). The proportions of CD4 positive splenocytes were lower in the 3 SCID mouse recipient groups (3, 4A, 4B) than in the normal BALB/c recipients. However, only the SCID mice in group 4B, which received CD8 depleted splenocytes, were infected (ip) with MHV-68 and treated with 4'-s-EtdU, had a significantly lower proportion of CD4 T-cells than the other SCID groups. Across the time course, the group 4B average CD4 count was 4.7% (+/- 1.9), as compared to 9.4% (+/- 2.9), 9.1% (+/- 2.7) and 13.1 (+/- 4.9) for groups 3, 4A and normal BALB/c recipients respectively (P=0.005, 0.005 and 0.022 respectively by Student T-test). Significant numbers of CD8 T-cells were also detected across the time course in the non-CD8 depleted mice (groups 3). The levels of CD8 positive splenocytes were below the limit of detection at every time point in the CD8-depletey mice (groups 4A and 4B), with the exception of the 2 group 4A mice sampled at day 7 post adoptive transfer, the CD8 counts for which were 1.2% and 1.6% (*see figure 5.3.4B*). The proportion of IgM positive splenocytes was low in all the SCID mice recipients (groups 3, 4A and 4B) compared to both the normal BALB/c recipients and the donor splenocytes, at every time point tested (*see figure 5.3.4C*). The null cells were the largest splenocyte sub-population present in the

Figure 5.3.4

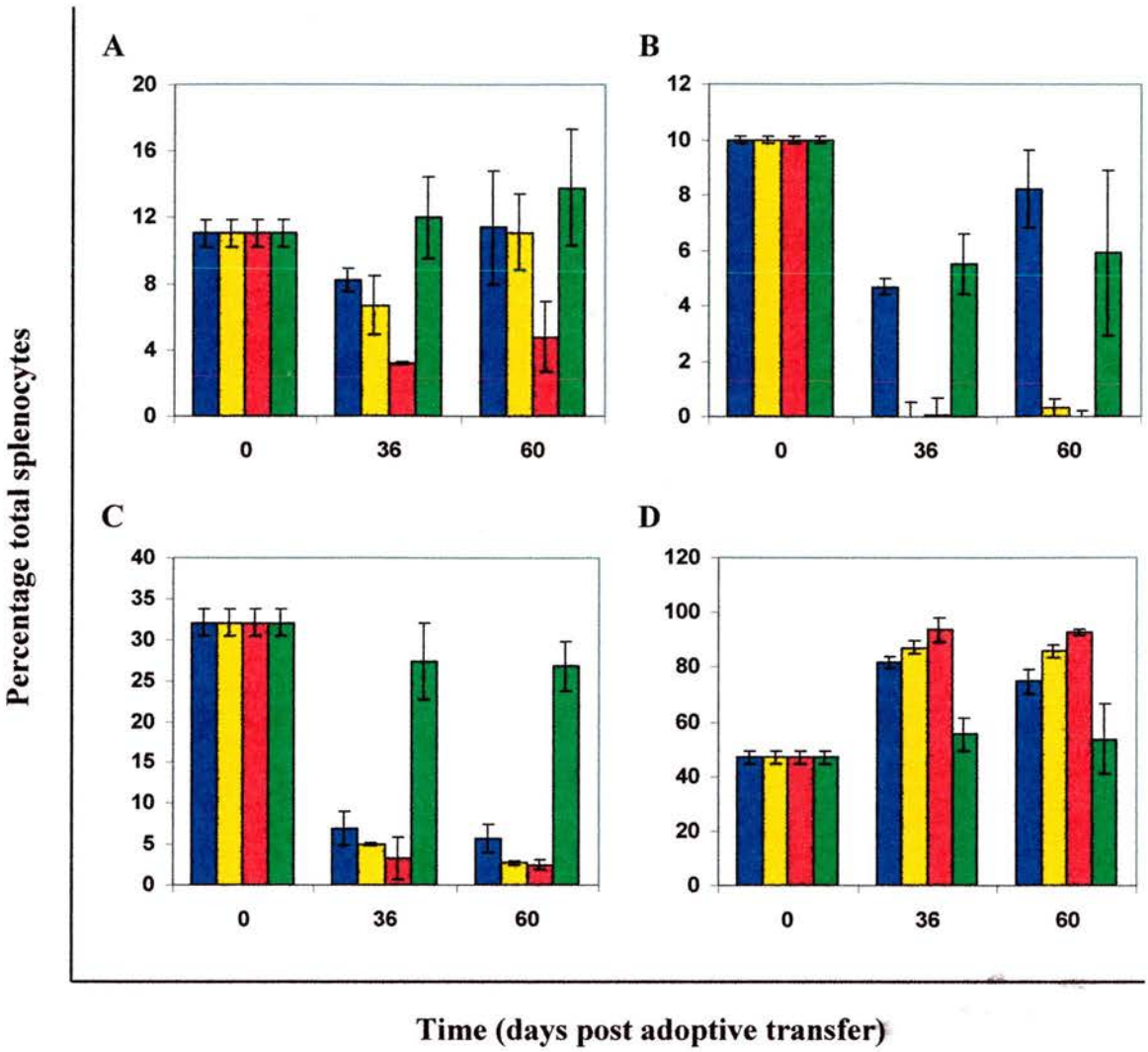


Figure 5.3.4 The relative proportions of CD4 (A), CD8 (B), IgM (C) and null (D) positive splenocytes observed in groups 3, 4A, 4B and 6 splenocyte adoptive transfer recipient mice. Groups 3, 4A and 4B (blue, yellow and red, respectively) comprised of SCID mice and group 6 (green) comprised of BALB/c mice. The mice of each group received $4x10^7$ splenocytes (pooled from BALB/c mice at day 11 post (ip) infection) via intra-peritoneal injection (0.4ml per mouse). Groups 4A and 4B received CD8 T-cell depleted splenocytes. Group 4B mice were infected with $4x10^4$ pfu MHV-68, via intra-peritoneal injection, 24 hours post adoptive transfers and then placed on drinking water supplemented with 4'-s-EtdU (0.3mg/ml) from day 6 post adoptive transfer. The proportions of CD4, CD8 and IgM positive splenocytes was determined by FACS analysis and the number of null cells was calculated as the cumulative proportion of CD4, CD8 and IgM negative cells. FACS analysis was carried out on the splenocyte preparations from the mice sampled on days 36 and 60 post adoptive transfer (2, 2 and 3 mice respectively per group). The day 0 post adoptive transfer value represents the adoptive transfer splenocytes. The data is represented by arithmetic means and the error bars represent the standard deviations. The limit of detection was 1%.

reconstituted SCID mice. The average proportion of null cells across the time course was 91.3% (+/- 4), 86.3% (+/- 2.1), 78.6% (+/- 4.7) and 54% (+/- 9.6) for the group 3, 4A, 4B and normal BALB/c mice respectively (*see figure 5.3.4D*).

5.2.9 **Virus latency in SCID mice reconstituted with splenocytes from MHV-68 infected BALB/c donors.**

Infectious centre assays were carried out on the spleen cells on all the mice sampled (*see figure 5.3.5*). At day 7 post adoptive transfer, the infectious centre levels in the spleen was relatively high in the CD8 non-depleted SCID mice (groups 3) and the normal BALB/c recipients. However, only 1 of the 2 mice sampled from each CD8 depleted group (4A and 4B) gave rise to detectable infectious centres (both positive mice being on the limit of detection for the assay). At day 36 and 60, infectious centres were difficult to detect in the SCID mice. Only 2 / 6 SCID mice, sampled on day 36 post adoptive transfer (1/2 group 3 and 1/2 group 4A mice) and 1 / 12 SCID mice, sampled at day 60 post infection (1/4 group 4A mice) gave rise to detectable infectious centres. At day 37 and 60 post adoptive transfer 2/2 and 2/4 normal BALB/c mouse recipients gave rise to infectious centres. There was no significant increase in spleen cell numbers or lymphoma development, in any of the mice sampled across the time course (*data not shown*).

5.3.1 **The effect of MHV-68 infection on SCID mice not reconstituted with spleen cells.**

The SCID mice not reconstituted with splenocytes (groups 5A and 5B) were monitored daily for signs of illness. The non 4'-s-EtdU treated SCID mice (group 5B) all showed signs of illness on day 12 post infection, and 3 of the 4 mice were dead by the day 13 post infection (the fourth mouse was killed due to signs of severe distress). The 4'-s-EtdU mice did not show signs of illness through out the experimental time course and were killed at day 59 post infection.

Figure 5.3.5

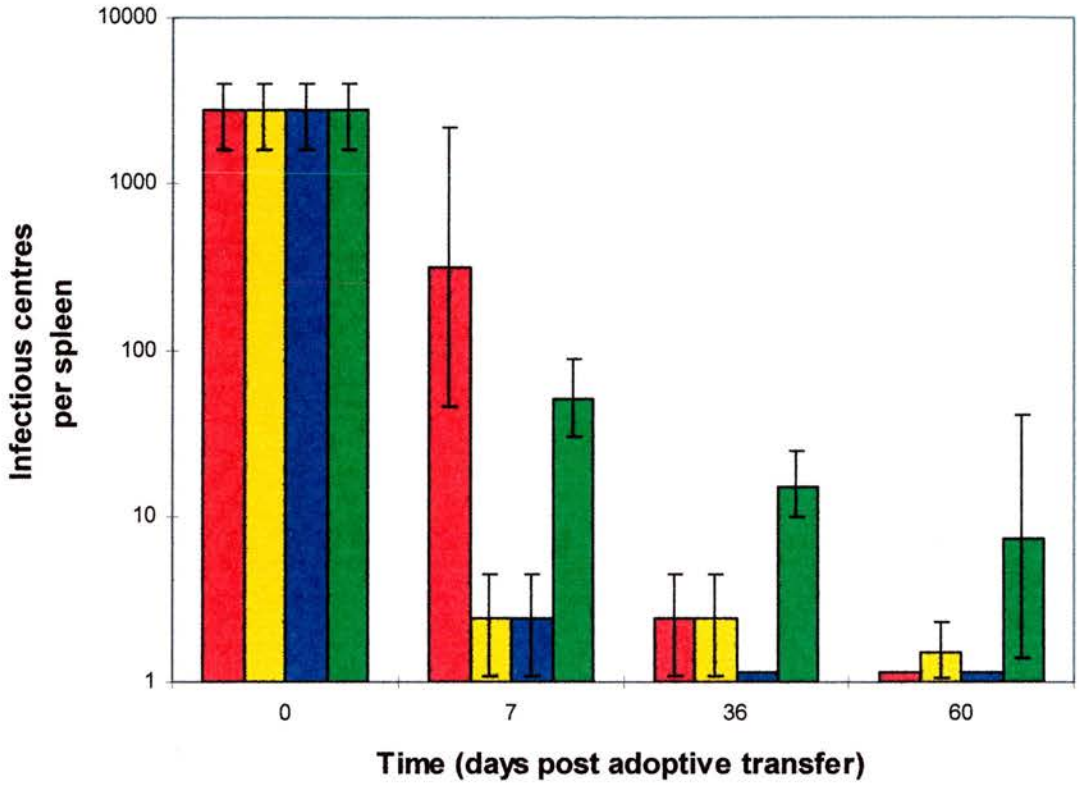


Figure 5.3.5 The levels of viral latency, as determined by infectious centre assay, ii groups 5, 6, 7 and 10 of the splenocyte adoptive transfer recipient mice. Groups 5, 6 and 7 (blue, yellow and red, respectively) comprised of SCID mice and group 10 (green) comprised of normal BALB/c mice. The mice within each group received $4x10^7$ splenocytes (pooled from infected BALB/c mice) via intra-peritoneal injection (0.4ml per mouse). Groups 6 and 7 received CD8 T-cell depleted splenocytes. Groups 7 mice were infected with $4x10^4$ pfu MHV-68, via intra peritoneal injection, 24 hours post adoptive transfers and then placed on drinking water supplemented with 4'-s-EtdU (0.3mg/ml), from day 6 post adoptive transfer. Mice were sampled from each group on days 7, 36 and 60 post adoptive transfer (2, 2 and 4 mice respectively). The day 0 post adoptive transfer value represents the number of latently infected cell injected (ip) into each mouse. The data is represented by geometric means and the error bars represent the standard deviations.

5.3.2 The effect of CsA on the MHV-68 infection in C57BL/6 mice.

The CsA treatment of the mice did not appear to directly affect the proportions of B or T-cells in the spleens of either the infected or uninfected mice. There was no significant differences in either the proportions or total numbers of the different lymphocyte sub-sets observed in the spleens of the mock infected mice (at the gross level), before and after CsA treatment. Likewise, there were no significant differences in either the proportions or total numbers of the different lymphocyte sub-sets observed in the spleens of the CsA treated and non-treated MHV-68 infected mice (when compared at the same time point). However, the CsA treatment did appear to directly affect the viral latency levels. After 6 days of CsA treatment a significant increase in splenic infectious centres was observed, as compared to both the titres seen 1 day prior to treatment, and to the untreated infected mice sampled in parallel of the treated mice. This effect appeared to be transient since by day 37 post infection (10 days after the last CsA injection) the infectious centre levels, of both the treated and untreated mice had, decreased to barely detectable level.

The CsA treatment was started after the acute infection had subsided and directly after peak splenic latency titres, based on previous observations (Sunil-Chandra *et al*, 1992 and Nash *et al*, 1996). The infected mice were therefore undergoing splenomegaly when treatment started. This was shown by the significant increase the number splenocytes observed at day 17 post infection, in the infected mice, as compared to the mock infected mice. There were marked differences in the proportions of the splenic lymphocyte sub-populations detected in the infected and uninfected mice. The infected mice contained a significantly higher proportion of CD8 T-cells and a significantly lower proportion of B-cells. Whilst the numbers of splenocytes was significantly higher, there were significantly more CD4, CD8 and B220 positive cells in the infected than the uninfected mice. After the increase in splenocytes reduced to a non-significant level, the number of B-cells per spleen

became indistinguishable from the number seen in the uninfected mice. However, the numbers of CD8 and CD4 T-cells remained significantly higher up to day 37 post infection.

5.3.3 **The apparent absence of LPD development in long-term MHV-68 infected mice.**

After a period of 2 years the mice were killed, however none appeared to have developed grossly enlarged lymphoid organs indicative of LPD development as previously described by earlier studies (Sunil-Chandra *et al*, 1994). Clearly this experiment failed to reproduce the finding of Dr Sunil-Chandra *et al*, (1994), who reported 9% of MHV-68 infected mice (predominately BALB/c, but including C57BL/6 mice) developed LPD over a period of 9 months to 3 years post infection. It was also reported that 60% of mice treated with CsA, early on in the infection went on to develop LPD over a 12 month period.

Perhaps the most obvious explanation for the difference is that the previous study was predominately carried out with BALB/c mice where this study used C57BL/6 mice. It is possible that the BALB/c mice are more susceptible to MHV-68 associated LPD development. It is also possible that differences in husbandry may have affected the susceptibility to lymphoma development. However, a number of differences in the methodology could also account for the differences. The mice in the previous study were infected via the intra-nasal route where as the mice in this study were infected via the intra peritoneal route. Subtle differences in the initial establishment of viral persistence or the relative severity of the initial acute infection at discrete sights may play a role in the development of malignancies or priming of the immune system. The CsA treatments were also slightly different. In the previous study weekly ip injections of CsA (50mg/kg) were given from day 4 to day 38 post infection. In this experiment the injections were twice weekly starting at day 18 post infection and finishing on day 27 post infection. Although this regime may have incorrectly induced long-term sensitivity (or immune tolerance) to tumour

development, it would not have affected the untreated infected mice.

5.3.4 **The adoptive transfer of splenocytes into SCID mice.**

The number of splenocytes detected in the spleens of the SCID adoptive transfers was compared to the total number injected into each mouse. The splenocyte adoptive transfers were injected in the SCID mouse recipients by the intra peritoneal route. Injection by the intra venous route may have been a more efficient route but due to the technical difficulties of working in an isolator, it was not possible. The CD4 and CD8 T-cells appeared to transfer more efficiently than the B-cells and were detected in higher numbers at every time point studied, in the SCID recipients. IgM positive B-cells represented approximately 5% of the total splenocytes present in the SCID recipient mice. The CD8 T-cell depletions appeared effective in both the first (pilot) and second experiment, as determined by splenocyte FACS analysis. However, splenocyte adoptive transfers to the groups which received the CD8 depleted splenocytes appeared to be less efficient, than the non-CD8 depleted splenocytes. The total splenocyte count tended to be lower in the CD8 depleted groups as did the CD4 T-cell and B-cell counts. However, significant numbers of donor splenocytes were generally detectable in the spleens of all the recipient SCID mice, at all the time points studied.

5.3.5 **The levels of viral latency observed in SCID mice 21 days after adoptive transfer.**

In the pilot experiment, latent virus was detected (by infectious centre assay) in 11/12 of the mice sampled at day 21 post adoptive transfer. The BALB/c splenocyte donors were treated with 4'-s-EtdU, at doses previously shown to inhibit productive viral replication *in vivo*, for at least 4 days prior to adoptive transfer. It is therefore reasonable to assume that no free virus was transferred with the splenocytes, hence any virus transferred to the SCID mice came from latently infected cells. The number of splenic infectious centres detectable in the SCID mice recipients, were

generally at least 10 fold lower than the total number present in the adoptive transfer. Despite the infectious centre levels being higher in the CD8 depleted mice, the differences were not significant. No apparent viral reactivation occurred at the gross level in the SCID recipients since no virus was detected in either the lungs, spleen or adrenal gland, all of which have been previously described as sites of acute viral productive replication (Sunil-Chandra *et al*, 1992). Further more there was no apparent difference between the mice treated with 4'-s-EtdU and those that were not, with respect to splenic infectious centres.

5.3.6 **The levels of viral latency observed in SCID mice splenocyte recipients over a 60 day time course.**

EBV has been shown to be more oncogenic in humanised SCID mice when added exogenously. Relatively high levels of infectious centres were seen at day 7 post adoptive transfer, in the mice who received splenocytes from uninfected donors and then infected (ip) with MHV-68. Although infectious virus assays were not carried out on the splenocytes, the numbers of infectious centres observed in the 4'-s-EtdU treated mice was essentially the same as the non-4'-s-EtdU treated mice. However, by day 36 the levels of viral latency had decreased to below the limit of detection in for the majority of the SCID mice recipients (as was the case at day 60 post adoptive transfer). Again higher levels of infectious centres were observed in the CD8 depleted mice than the non-depleted mice, but with only 2 mice per group sampled, the significance of this observation is difficult to determine.

The mice that received splenocyte adoptive transfers from infected mice followed a similar pattern. At day 7 post adoptive transfer, infectious centres could readily be detected in the non-CD8 depleted SCID recipients and the normal BALB/c recipients. Infectious centres could not be readily detected in the CD8 depleted recipients, even those super-infected with MHV-68 and then placed on 4'-s-EtdU. The implication from this is that either the latently infected B-cells failed to traffic to the spleen or suffered a loss of viability, presumably due to the anti-CD8 antibody. It

has been observed in the past that splenocytes can form large non-specific clumps in the presents of anti-CD8 antibody, once warm. Infectious centres could not readily be detected in the SCID mice recipients, at the latter time points. Interestingly infectious centres could be detected in half the normal BALB/c mice tested (2/4) at day 60 post adoptive transfer, when infectious centres could not be detected in any of the parallel SCID mouse group (0/4 non-CD8 depleted recipients). However, whether this result means that latently infected B-cells survive better in a normal immunocompetent mouse than a SCID mouse, would require a far more thorough investigation, specifically designed to test this hypothesis.

5.3.7 **The oncogenesis of MHV-68 in SCID mice recipients.**

There was no obvious signs of lymphoma development associated with the lymphoid organs in any of the SCID mice. Neither was there any gross increase in the number of splenocytes. EBV positive PBLs generally give rise to LPD after 1 month post adoptive transfer. Clearly this was not observed in either the SCID mouse recipients of either uninfected splenocytes, which were then infected or splenocytes from infected mice. This could have been due to insufficient time allowed for lymphoma development, however it could equally be due to the authenticity of this model. The incidence of LPD development in humanised SCID mice (or in cotton top tamarins) is far higher than is ever experienced in EBV positive immunocompromised or immunosuppressed humans. Other inconsistencies exist, such as the fact that LPD develops in humanised SCID mice despite the presence of the cells (such as CD8 T-cells) believed to be responsible for preventing LPD development in the original donor. Treatment with immunosuppressants such as CsA, greatly reduces the incidence of LPD development in the SCID mice (Veronese *et al*, 1992), where in humans it could be considered a prerequisite.

5.3.8 **The MHV-68 infection of SCID mice.**

All the mice were inffected with $4x10^4$ pfu MHV-68 via the intra peritoneal route of

infection. The infection was lethal in the SCID mice which were not treated in any other way. Although time did not permit analysis of the tissues retrieved from the mice by post-mortem. However, death was probably caused by disseminated infection since the mice treated with 4'-s-EtdU showed no signs if illness for up to day 60 post infection. The mice that received splenocyte adoptive transfers also failed to develop disease from the infection. Interestingly the mice that received CD8 depleted splenocytes likewise failed to develop disease. This is in direct contrast to the intra-nasal infection of BALB/c mice, which when CD8 depleted using the same monoclonal antibody as was used in this experiment, have been shown to develop lethal disease (Ehtisham *et al*, 1993). This may be accounted for by the fact that the SCID mice were infected with 10 fold less virus, than was used in the CD8 depletion study. However, the intra-peritoneal route of inoculation generally gives rise to a more severe acute infection in immunocompromised mice than more natural routes of infection (Dutia *et al*, 1997, Sarawar *et al*, 1997 and Weck *et al*, 1997). The adoptively transfer process may also have affected the immune response to infection, mediated by the splenocytes, or there affects on the non-specific immune system of the SCID mice. Further research will be required to resolve this inconsistency.

Thesis summary

A number of gammaherpesviruses have now had there genomes either partially or completely DNA sequenced. This has allowed cross-species comparisons between different gammaherpesviruses, with respect to genetic conservation rather than comparisons based purely on genomic architecture and cellular tropism. MHV-68 can clearly be identified along with the other gamma-2 herpesviruses, encoding homologues for CRP, cyclin D, GCR and the BHV IE1 / KSHV K3 protein (Virgin *et al*, 1997). Also common to gammaherpesviruses, MHV-68 encodes a homologue to the EBV R transactivator and a Bcl-2 homologue (Virgin *et al*, 1997). Despite the genetic similarity of MHV-68 to other gammaherpesviruses, MHV-68 is not associated with the development of MCF-like diseases, as is seen in AHV-1 and OHV-2 infections of cows and deer respectively (see Table 1.4). Likewise, MHV-68 does not acutely transform either B or T lymphocytes *in vitro*, like EBV or HVS (Pope *et al*, 1968 and Medveczky *et al*, 1984). MHV-68 does not encode homologues to the transformation determinants of either EBV or HVS or host interleukin molecules. However, the pathology of the MHV-68 infection of mice does share much in common with other B-cell tropic gammaherpesviruses, such as the patterns and titres of latently infected B-cells and the splenic lymphoproliferations observed post acute infection (Sunil-Chandra *et al*, 1992A and B, Ehtisham *et al*, 1993 and Nash *et al*, 1994 and 1996).

This study was aimed at determining the oncogenic potential of MHV-68 and to elucidate the mechanisms by which MHV-68 initiates infection, establishes latency

and induces splenomegaly in inbred mice strains. The method used to study the early events of the MHV-68 infection of mice, was achieved using a new and highly potent inhibitor of herpesvirus replication termed 4'-s-EtdU. 4'-s-EtdU is a deoxy uridine / thymidine homologue which has been shown to inhibit the replication of alphaherpesviruses such as HSV and VZV (Rahim *et al*, 1996). In this study the effect of 4'-s-EtdU on the replication of MHV-68 in a number of different *in vitro* cell lines was examined. Initially the potency of 4'-s-EtdU was determined by plaque reduction (EC_{50}) assay, using the anti-viral ACV as a comparative standard. MHV-68 has been shown previously to be sensitive the ACV (Sunil-Chandra *et al*, 1994B), but in this study, over 10 times less potent than 4'-EtdU. The EC_{50} of 4'-S-EtdU was found to be 35ng/ml (0.13μM) and 450 ng/ml (2μM) for ACV. The effects of 4'-s-EtdU on the replication of MHV-68 was studied in 3 different cell lines, known to be infected by MHV-68. The 3 cell lines were chosen because each supported a distinct pattern of virus replication. The myeloma derived NS0 cell line, being of mouse B-cell origin, supports a predominantly latent MHV-68 infection (Sunil-Chandra *et al*, 1993). Although persistently infected cultures always contain productively infected cells, the culture never reaches crisis, with the number of productively infected cells remaining constant over time. Both the fibroblastoid BHK cell line and the transformed glial MGC-7 cell line support lytic MHV-68 replication, leading to plaque formation and an infected culture inevitably undergoing crisis. Plaque formation in the MGC-7 cells takes significantly longer than in BHK cells (Terry *et al, personal communication*).

Persistently infected NS0 cultures where treated with 4'-s-EtdU at the minimum dose

required to prevent plaque formation in BHK cells (over 4 days), 200ng/ml and at a saturating dose, 10 fold higher, 2μg/ml. Both concentrations of 4'-s-EtdU significantly reduced the amount of productive viral replication detected in the cultures by infectious virus assay. No infectious virus was detected in the cultures after the first week of 4'-s-EtdU treatment at 2μg/ml. Likewise MHV-68 antigen expression was also eliminated from the persistently infected cultures treated with 2μg/ml 4'-s-EtdU. Immunofluorescent staining was carried out on the treated cultures using both anti-MHV-68 rabbit hyperimmune sera and sera taken from MHV-68 infected mice. Both seras failed to detect viral antigen expression in the 4'-s-EtdU treated cultures. Treatment at the lower of the two 4'-s-EtdU doses, 200ng/ml, failed to eliminate infectious virus and hence productive viral replication from the persistently infected NS0 cultures. However, the titres of infectious virions present in the culture was decreased 1000 fold, to levels that were on the limits of detection. The conclusions from these results were that 4'-s-EtdU at 200ng/ml significantly reduced the rate of MHV-68 productive replication but did not prevent it. Although 4'-s-EtdU at both 200ng/ml and 2μg/ml were sufficient to prevent plaque formation in BHK monolayers, over a 4 day period, only at 2μg/ml was the 4'-s-EtdU completely inhibiting productive viral replication in the cells.

Co-cultivation of the MHV-68 infected NS0 cells from the 4'-s-EtdU treated cultures over the time course, showed that despite eliminating all detectable infectious virus from the cultures it was not possible to eliminate infected cells from the cultures. The infectious centre titre decreased in an inverted exponential manner for the

duration of treatment with both doses of 4'-s-EtdU. This result implied 4'-s-EtdU inhibited the ability of latently infected cells to reactivate and undergo productive replication after withdrawal of treatment. At 200ng/ml 4'-s-EtdU, this effect was relatively short lived since on withdrawal of treatment, both infectious virus and infectious centre titres returned to the pre-treatment titres. The suppression of viral reactivation was far more pronounced in the culture treated with 2μg/ml 4'-s-EtdU. The infectious centre titres remained for the duration of the experiment (23 days) at the level observed just prior to treatment withdrawal. Productive virus replication was detected 3 days after withdrawal of the 4'-s-EtdU treatment, however, again titres failed to return to the pre-treatment titres. The suppression of viral reactivation observed in the 4'-s-EtdU treated cultures was not due to a lower number of MHV-68 infected NS0 cells present in the cultures. PCR analysis carried out on the genomic DNA derived from cells cloned from both treated and untreated NS0 cultures demonstrated that the number of cells harbouring viral genome remained insignificantly altered. It is also unlikely that the suppression observed was due to viral genomes becoming non-viable, since infectious virus was recovered from the MHV-68 genome positive cell lines cloned from the 4'-s-EtdU treated cultures. The suppression of reactivation observed could be similar to the effects of FCV on the HSV reactivation *in vivo* (Field *et al*, 1995a & b and Thackray *et al*, 1986 and 1987).

The 4'-s-EtdU treatment of MHV-68 infected MGC-7 cells gave surprisingly similar results to the treatment of the infected NS0 cells. Infection of MGC-7 cells at a MOI of 1 pfu MHV-68 / cell, would normally result in the culture undergoing crisis within 2 to3 days. However, MGC-7 cultures infected at a MOI of 1 and then treated with

4'-s-EtdU at either 200ng/ml or 2μg/ml, despite an initial toxicity, failed to under go crisis. Treatment at 200ng/ml failed to protect the cultures for more than 2 to 3 weeks, presumably due to low level productive viral replication. However, 4'-s-EtdU treatment of the infected MGC-7 cells at 2μg/ml protected the infected cultures for over 2 months of continual culture. The treated cells were MHV-68 antigen negative by immunofluorencent staining using both anti-MHV-68 rabbit hyper-immune sera and sera taken from late time point MHV-68 infected mice. However, on withdrawal of treatment, the MHV-68 harboured in the culture reactivated, as determined by anti-MHV-68 antigen immunofluorecent staining of the cells. All the cells had died by day 10 post treatment withdrawal and were MHV-68 antigen positive. Hence on withdrawal of treatment the cells were killed by lytic viral replication. By cloning the MHV-68 infected 4'-s-EtdU treated MGC-7 cells, it was determined that 1/3 of the cells within the culture harboured MHV-68 virus. These results were interesting since MHV-68 could clearly maintain genomic copy number, in the apparent absence of productive replication in a cell line which was previously believed to support only lytic viral replication.

Treatment of MHV-68 infected BHK cells with 4'-s-EtdU at 2μg/ml failed to protect the cultures at high MOI. Treated cells infected at a MOI of 4 were mostly dead by 5 days post infection and only partial protection was observed in the cells infected at an MOI of 0.4. However, short term protection up to 2 weeks, was observed in the cultures infected at an MOI of 0.04 or less. Again the virus was not lost from these cultures because after 2 weeks following withdrawal of treatment viral plaques developed in the cultures leading to all the cells dying. The studies on the effect of

4'-s-EtdU treatment of MHV-68 infected BHK cells was greatly hampered by the spontaneous outgrowth of 4'-s-EtdU resistant virus which generally arose after 2-3 weeks of treatment.

Polyclonal MHV-68 preparations were generated from the 4'-s-EtdU treated MHV-68 infected NS0 cultures after 47 days of continual treatment. However, no increased sensitivity to 4'-s-EtdU was observed by EC_{50} assay. A polyclonal MHV-68 preparation was also generated from a long-term 4'-s-EtdU treated MHV-68 infected MGC-7 culture, which spontaneously under went crisis whilst still being treated. The polyclonal MHV-68 preparation was found to be resistant to 4'-s-EtdU and MHV-68 isolates were cloned and partially characterised. Preparations of 4'-s-EtdU resistant MHV-68 were also generated from the infected BHK cultures which spontaneously developed plaques during treatment. It was interesting to observe that resistant variants only arose from the infected cell lines which under normal circumstance support lytic virus replication. Also that spontaneous generation of 4'-s-EtdU resistant variants took much greater time to emerge from the infected MGC-7 cells than the infected BHK cell. Since MHV-68 productively replicates slower in MGC-7 than BHK cells, it could be hypothesised that the time it takes for development of resistant variants is inversely linked to the rates of productive virus replication supported by the infected cell line. However, further research would be required to verify this hypothesis. Many more cell lines which support the latent / persistent / lytic replication of MHV-68 would have to be studied. Also, a longer duration of treatment would be required for cell lines such as NS0 so as to give them time, if it is at all possible, to spontaneously develop 4'-s-EtdU resistant virus

variants.

The 4'-s-EtdU resistant MHV-68 isolates cloned from the MGC-7 culture, termed MERV isolates, were found to be resistant to 4'-s-EtdU up to 10μg/ml. At the gross level, the MERV isolates were indistinguishable from wild type MHV-68 with respect to rates of plaque formation in BHK cells and virus titres, measured in pfu, recovered from BHK cells infected at a MOI of 0.01. The MERV isolates also were indistinguishable from wild type virus with respect to sensitivity to ACV, when measured by EC_{50} assay. The results strongly implied that resistance to 4'-s-EtdU was not due to the deletion of the TK gene but was due to point mutations in either the TK gene or the virus encoded DNA polymerase. However, the mechanism by which MHV-68 becomes resistant to 4'-s-EtdU, without affecting ACV sensitivity can not be resolved until the DNA polymerase and the TK encoding genes of the MERV clones are cloned and sequenced.

In vivo, the oral treatment of mice with 4'-s-EtdU at a dose of 1 mg/mouse/day was found to be sufficient to not only reduce the productive replication of MHV-68 but to prevent the productive replication of MHV-68. The evidence for this came from a number of observations. Treatment of mice at 1mg/mouse/day eliminated infectious virus from the lungs of mice infected via the intra-nasal route during the acute infection, when determined by infectious virus assay. Prophylactic 4'-s-EtdU treatment of mice, infected via the intra-nasal route, prevented not only the production of MHV-68 specific antibodies, by the mice during prolonged courses of treatment, but also prevented dissemination of virus from the lungs to other parts of

the body, such as the spleen, despite infection being established.

Initial *in vivo* experiments were carried out to determine the effect of 4'-s-EtdU (1mg/mouse/day) treatment starting 3 days post infection. The treatment resulted in the acute infection of the lung being rapidly resolved, following intra-nasal infection, when determined by infectious virus assay. Treatment also caused a delay in the accumulation of latently infected B-cells in the spleens of the treated mice, when determined by infectious centre assay. However, 4'-s-EtdU treatment failed to have an long term effect on latent virus titres in the spleen and also failed to prevent the development of the post acute splenomegaly. The conclusions from these results were that although severity of the primary acute infection of the lungs may have influenced the number of B-cells that initially became latently infected, it did not directly effect the development of splenomegaly (post acute lymphoproliferitive disease) and had no long-term effect on the levels of viral latency in the spleen. More over, on the assumption that by approximately 24 hours after initiation of treatment (day 4 post infection), productive viral replication in the mice was arrested, we further concluded that productive virus replication did not play a direct role in the development of splenomegaly and was not required for long term maintenance of B-cell latency. This was concluded since latently infected B-cells can not generally be detected in the spleen of mice until days 6 to 7 post infection and along with the development of splenomegaly, titres do not peek until days 12 to 15 post infection. It could be argued that 4'-s-EtdU resistant variants spontaneously arose *in vivo* and so allowed productive virus replication to occur in the presence of the treatment. However, virus preparations were generated by co-cultivation of lung

tissue and splenocytes though out the time course and no decreased sensitivity to 4'-s-EtdU was observed in any mouse at any time point. These results are in full agreement with the observation made with the attempted treatment of patients with EBV post acute infectious mononucleosis (Pang *et al*, 1997 and Niedobitek *et al*, 1997).

A further observation in this experiment was that MHV-68 could be far less readily reactivated from the lungs of the infected mice during 4'-s-EtdU treatment than the untreated mice. Because MHV-68 could be reactivated from the splenocytes at late time points of the treated mice at the same efficiency as the untreated mice, it is unlikely that viral persistence in the lungs was due to latently infected B-cells present in the tissue. Further more, the results imply that the viral persistence in the lungs of mice involved low level chronic virus replication but that chronic replication did not have a role in viral persistence in the spleen. The reason MHV-68 was not detected in the lungs of untreated mice at the late time points by direct plaque assay, was presumably due virus titres being below the limits of detection. Likewise, the 4'-s-EtdU treatment of the mice further reduced the virus titre to below the co-cultivation assay limit of detection, since on withdrawal of treatment virus was once more detected in the lungs of the formally treated mice.

The next set of *in vivo* experiments were carried out to determine the effect of prophylactic 4'-s-EtdU treatment (1mg/mouse/day) starting 2 days prior to intra-nasal infection. The treatment resulted in there being no detectable productive virus replication in the lungs, during the times normally associated with the acute lung

infection. Virus also failed to disseminate to the spleen in the treated animals, as determined by both co-cultivation assay and nested PCR. Further more, the treated mice failed to under go splenomegally or produce MHV-68 specific antibodies. However, infection was established in the prophylactically treated mice since on withdrawal of treatment, viable virus was detected first in the lungs and then later in the spleen. PCR and nested PCR analysis on DNA extracted from the lungs and spleens of the treated, untreated and treatment withdrawn mice clearly demonstraighted that viral infection was established in the lungs of the mice following intra-nasal infection even in the prophylactically treated mice. However, there was significantly more virus genome present in the lungs of the untreated mice than the treated. Viral genomic DNA remained present in the lungs of the treated mice and remained absent in the spleen of mice at all time points sampled (up to 54 days post infection). Upon withdrawal of treatment, the levels of viral DNA increased in the lungs of the mice and over a period of weeks became detectable in the spleen. Dissemination of the virus from the lungs to the spleen coincided with the production of MHV-68 specific antibodies by the infected mice.

From these experiments it was concluded that productive virus replication was not required for the long-term establishment of the MHV-68 infection in the lungs of mice, inoculated via the intra-nasal route. At the cellular level, *in vitro* experiments carried out earlier in this study had already shown that MHV-68 readily infected NS0, MGC-7 and BHK cells, in the presence of saturating doses of 4'-s-EtdU. It was further concluded that viral persistence in the lungs was not dependent on either productive virus replication or the existence of latently infected B-cells. However, as

was concluded from the experiment in which 4'-s-EtdU treatment was initiated day 3 post infection, productive or chronic virus replication was responsible for a large proportion the persistent virus present in the lungs of infected mice under normal circumstances. There were striking similarities between the viral persistence in the lungs of mice during 4'-s-EtdU treatment and the viral persistence in the *in vitro* cell lines also during treatment. This study has provided clear evidence, both *in vitro* and *in vivo*, that in the presence of high doses of 4'-s-EtdU and the apparent absence of productive viral replication, MHV-68 can persist in cell lines and tissues (of non lymphoid origin) which are usually associated with lytic or productive virus replication.

Evidence from the study on the effects of prophylactic 4'-s-EtdU treatment on the intra-nasal MHV-68 infection of mice also showed that productive viral replication had a major role in the aetiology of the MHV-68 infection. It was concluded that productive virus replication was essential for virus dissemination to the spleen and hence essential for the initial establishment of viral latency in B-lymphocytes. Also, productive virus replication was essential for development of splenomegally. Earlier experiments had already established that productive virus replication does not play a direct role in inducing splenomegaly, however it was required to initiate the events which lead to the development of splenomegaly. This is presumably due to productive replication being required to establish latent infections of B-cells and so supports the work carried out on the pathogenesis of the MHV-68 infection of μMT mice (Usherwood *et al*, 1996b).

A further experiment was also carried out on the effects of prophylactic 4'-s-EtdU treatment on the MHV-68 infection of mice. In this experiment the virus was inoculated via the intra-peritoneal route. Again the infection failed to disseminate to the same degree as with the untreated mice. Productive virus replication was again not detected in the mice during treatment and the mice failed to develop splenomegaly. A number of the treated mice did establish viral latency in the spleens however, latent virus titres were far lower than in the untreated mice as determined by both infectious centre assay and PCR. In the majority of the treated mice, virus had not established an infection in the lungs and half the mice failed to establish infections in either the lungs or the spleen, as determined by nested PCR. Again all the mice appeared to become infected since on withdrawal of the 4'-s-EtdU treatment viral genome was detected in both the lungs and spleen of all the mice.

It was hard to draw many conclusions from this experiment since this study was much smaller than the other *in vivo* studies and the MHV-68 infection has been less well described when initiated via the intra-peritoneal route as opposed to the intra-nasal route. The effects of the 4'-s-EtdU treatment were perhaps far greater than might be expected from a systemic route of challenge such as the intra-peritoneal route of infection. The study did clearly demonstrate the essential role of productive virus replication for viral dissemination. However, the efficiency of dissemination along with the levels of viral genome present in tissues appeared to have a far greater degree of variability in the treated mice infected via the intra-peritoneal route than via the intra-nasal route. It was interesting to observe that viral latency was

established in some of the prophylactically treated mice and the results implied a tissue other than the lungs or spleen may be harbouring persistent virus. The levels of latent virus observed in the spleens of the prophylactically treated mice was very low. However, further experiments would be required to determine whether it would have remained low over longer periods of time. Greater numbers of mice and a wide range of tissues would have to be studied to determine the extent of viral dissemination in the absence of productive viral replication, following intra-peritoneal inoculation. The results from this experiment implied that the development of splenomegaly might require either some productive virus replication or a threshold level of B-cells to become latently infected. This would probably best be resolved by inoculating MHV-68 at different doses into prophylactically 4'-s-EtdU treated mice via the intra-venous route. It could therefore be guarantied that high levels of viral latency would be established in B-cells in the absence of productive viral replication.

The final set of experiments undertaken in this study were to establish whether MHV-68 induced tumour formation in mice and if so the role of productive virus replication in the disease pathology. A number of gammaherpesviruses have transforming properties *in vitro* and are associated with the development of malignancies *in vivo* (see Table 1.4 for summery). Further more the prevalence of B-cell lymphoma development has been reported to be elevated in elderly MHV-68 infected mice compared to uninfected controls (Sunil-Chandra *et al*, 1994b). A large scale experiment was carried out where large groups of C57Bl/6 mice were either infected or mock infected with MHV-68. Half of the infected mice and all the

uninfected mice were then transiently immunosuppressed with cyclosporin A.. The treatment lasted for 10 days, initiated during splenomegaly, day 17 post infection. The treatment appeared to cause a transient elevation of latent MHV-68 titres in the spleens of the infected animals, which on withdrawal of treatment returned to base line levels. However, unlike the earlier report, which described up to 60% of mice developing LPD (Sunil-Chandra *et al*, 1994b), none of the mice went on to develop malignancies over the next 2 years.

Further experiments were carried out using lymphocyte repopulated SCID mice. Since SCID mice have no specific immunity they are highly prone to viral disease. SCID mice have been used extensively in the study of EBV induced LPD. Development of LPD can be developed in SCID mice by adoptively transferring PBL from EBV positive human donors LPD (Cannon *et al*, 1990 and Rowe *et al*, 1991). In an attempt to mimic this type of experiment, splenocytes were adoptively transferred from syngeneic MHV-68 donor mice into SCID mice. Despite the fact that latently infected B-cells were detected in the spleens of the SCID mice recipients 21 days after adoptive transfer, the virus titres were very low, even in the CD8 T-cell depleted groups.

A larger scale experiment was then carried out using SCID mice as recipients of MHV-68 infected splenocytes, using greater numbers of SCID mice over a longer time course. Splenocytes were adoptively transferred from MHV-68 infected mice into SCID mice as well as naive immunocompetent syngeneic mice. Further more, splenocytes were also adoptively transferred into SCID mice from uninfected mice

and then the SCID mice were infected with MHV-68 1 hour later. The mice were sampled up to 60 days after adoptive transfer but again, despite the fact that latent virus was detected in all the mice 7 days after transfer, none of the mice went on to develop LPD. Further more, the MHV-68 titres in the spleens of the SCID mice recipients appeared to substantially decrease over time and could not be detected in the majority of the mice 60 days after transfer. Latent virus appeared to persist better in the normal BALB/c recipients than in the SCID mice. CD8 T-cell depletion of the splenocytes did not appear to affect latency levels in the SCID recipients which is surprising given previous reports suggesting CD8 T-cells were essential for control of MHV-68 associated disease (Etisham *et al*, 1993). The study did however demonstrate at a specific immune system is essential to protect mice against lethal MHV-68 infection. SCID mice inoculated with MHV-68 via the intra-peritoneal route gave rise to a lethal infection, death occurring after 2 weeks. Death was presumably due to disseminating chronic virus replication since mice infected and then treated with 4'-s-EtdU from day 6 post infection did not show signs of disease until the experiment was terminated 59 days later. This series of experiments failed to provide evidence of MHV-68 involvement in the development of malignancies and hence it was concluded that if MHV-68 did have oncogenic potential it was not readily observed under the described experimental conditions.

To summarise the findings of the study presented in this thesis, productive virus replication is essential for dissemination of MHV-68 from the lungs of mice infected via the intra-nasal route to other mouse tissues such as lymphoid tissue. Productive virus replication also appeared to play a major role in viral dissemination even when

MHV-68 was inoculated by systemic routes such as the intra-peritoneal route. Productive virus replication is not required to establish infection in either cell lines *in vitro* or the host *in vivo*. Productive virus replication is not required to maintain levels of latently infected B-cells and does not play a direct role in the development of post acute non-malignant splenic lymphoproliferation. The lungs are a major site of MHV-68 persistence and persistence at this site is predominantly due to chronic virus replication. However, MHV-68 can persist in cell lines *in vitro*, and tissues such as the lungs, *in vivo*, in the prolonged absence of productive viral replication, despite being associated with productive virus replication and not with classical gammaherpesvirus latency.

The study highlights the importance of MHV-68 as a model by which the pathogenesis of gammaherpesvirus infections can be studied under controlled experimental conditions in the natural host. Although MHV-68 may not be a good model for the study of gammaherpevirus induced / associated malignancies it allows a unique means to study the early events in gammaherpesvirus infections. The study also highlights the potential of MHV-68 as a model for the study and testing of antiviral agents and therapies both *in vitro* and *in vivo*.

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